PHYLOGENY AND BIOGEOGRAPHY OF SOUTHERN AFRICAN LIMPETS IN THE GENUS *SIPHONARIA* IN THE CONTEXT OF A GLOBAL PHYLOGENY.

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DECLARATION

I hereby declare that the work on which this thesis is based is my original work, except where otherwise stated in the text. All sources used or quoted in the study have been indicated and acknowledged by way of citations and complete references. This thesis has not been submitted whole or in part for a degree at any other university.

Paula Marie de Coito

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CONTENTS

ACKNOWLEDGMENTS 1

ABSTRACT 2

Chapter 1
Introduction. 5
References 11-17

Chapter 2
Elucidation of *Siphonaria* species on the East Coast of Southern Africa. 18
References 70-74
Appendices 2.1-2.4 75-78

Chapter 3
***Siphonaria compressa*, South Africa's rarest endemic mollusc: one species or two?** 79
References 113-119
Appendices 3.1-3.2 120-121

Chapter 4
Integrating southern African species into a world phylogeny of *Siphonaria* species, and an analysis of biogeographic patterns in the genus. 122
References 158-167
Appendix 4.1-4.2 168-182

Chapter 5
Synthesis and Conclusion. 183
References 187

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Phylogeny and biogeography of southern African limpets in the genus *Siphonaria* in the context of a global phylogeny.

ABSTRACT

Abundant, diverse and ecologically important, the 'false-limpet' genus *Siphonaria* has a near world-wide distribution. However, arising from uniformity of shell shape among species and its plasticity within species, frequent synonymisations and changes of names have created uncertainty about the number of species that exist and the names that should be applied. In this thesis I pursue three lines of genetic research to address this. First, I resolve the species that exist in two South African complexes: the *Siphonaria carbo* complex and the *S. concinna/oculus* complex. Second, I examine what is currently accepted as *S. compressa* from the only two South African localities where it has been recorded, to determine if the two populations are conspecific. Finally, I compare the genetics of a selection of species from around the world to place the South African species within, and contribute to, a world phylogeny for the genus and analyse geographic patterns of clades emerging from this phylogenetic reconstruction.

With respect to the '*S. carbo*' complex, Teske *et al.* (2007), using mitochondrial and nuclear sequence data on *Siphonaria nigerrima*, *S. anneae*, and *S. tenuicostulata*, failed to discern distinct monophyletic lineages, and concluded that these species, together with *S. dayi*, are different colour morphs of a single species, merged under the name *S. nigerrima*. This contrasts with a previous systematic revision by Chambers & McQuaid (1994), which considered these to be four separate species. An added complication is that the name *S. nigerrima* has been synonymised with *S. carbo* by various authors and in a range of data bases.

Using phylogenetic analyses based on COI and 16S ribosomal RNA genes and incorporating additional GenBank sequences of east coast species, I confirm that *S. nigerrima* and *S. dayi* comprise a single species, to be merged under the name *S. nigerrima* because of its priority, and because examination of the type material of *S. carbo* indicates that it is unlikely that that species is present in South Africa. However, I also show that within the '*S. nigerrima* complex' there exist four or possibly even five clades that warrant recognition at a specific level, two of which constitute undescribed and unnamed species (nov. sp. 1 and nov. sp. 2) while the other three can be accommodated under the names *S. nigerrima* sensu stricto, *S. tenuicostulata* and *S. anneae*.

Using the same approach, I examined the distinction between *S. oculus* and *S. concinna*, which has become blurred in the literature, with only the latter being recognised by Teske *et al*. (2011). Both my molecular analyses and a consideration of shell features provide evidence that the two are distinguishable and valid species.

Siphonaria compressa was first described by Allanson (1958) from specimens found in Langebaan Lagoon on the West Coast of South Africa, where it is associated with the seagrass *Zostera capensis*. In 2005 a population of what was considered the same species was recorded in Knysna Lagoon on the South Coast. On the basis of COI and 16S

analyses coupled with shell morphometrics I found that the two populations have no shared haplotypes and are clearly diverged; IMa2 estimates indicate there has been no migration between these populations and the split between them occurred around 730 000 years ago. Evidence points towards recently expanding populations at both sites. Uni-, bi- and multivariate analyses of 13 morphometric variables revealed that the means for most variables are significantly different and clear differences in shell shape, size and weight exist between the two populations. I thus recognise two geographically delimited species, and in this thesis I distinguish the Knysna population as *Siphonaria* nov. sp. 3. The fact that the populations are considered separate species has important ramifications for conservation.

The third aspect of my thesis was to integrate the southern African species that I now recognised into the most recent global phylogeny of the genus (Dayrat *et al.* 2014), which focussed strongly on the Indo-West Pacific species and omitted all South African species. I sequenced 120 individuals collected from 51 localities including 11 southern African species – three of which I consider to be undescribed species – and 42 other species from around the world, nine of which were not previously included in any published phylogeny, thus adding a total of 20 species to the global phylogeny. In total, incorporating GenBank sequences, I analysed 272 mtDNA sequences (16S + COI, 1202 nucleotide characters) within a Bayesian framework. Sacoglossans were used as the outgroup as per Bouchet *et al.* (2017).

Similar to Dayrat *et al.* (2014), two major clades, A and B, were recovered. The South African species were not monophyletic but their positions were clear and distinct and did not influence the original division of the genus into two major clades. The inclusion of the South African species led to a rearrangement of the tree topology, especially within clade A, where all bar one of the South African species were recovered, with the the highly divergent *S. serrata* occurring in the second major clade B.

Within clade A all bar two of the South African species were retrieved in one clade. The exceptions were *S. compressa* and *Siphonaria* nov. sp. 3 which fell elsewhere within clade A and were more closely related to Pacific Ocean species. The contents of clade B remained similar to the previously published phylogeny with the exception of a significant rearrangement of the basal species. Although many species remain unnamed, six previously unidentified molecular units in the global phylogeny are identified here and assigned species names.

I also explored biogeographic patterns for all recognised species in the genus. There were strong latitudinal trends evident in species richness with high diversity in the lower latitudes, but with a dip at the equator, in contradiction to the prevailing paradigm of greatest diversity at the equator. Species with direct rather than planktonic development had both smaller geographic and smaller average latitudinal ranges.

Overall, this thesis encompassed a view of the genus from the small-scale within-species population genetics of the highly endangered and range-restricted *S. compressa* and its sister taxon *Siphonaria* nov. sp. 3, through the broader geographic delimiting of the species contained within two southern African species complexes, to the placement

of all the southern African species within a global context by updating the world phylogeny of the genus.

Chapter 1
Introduction

Background

This thesis deals with the molluscan family Siphonariidae (Class Gastropoda, Order Siphonariida), members of which are commonly known as 'false limpets'. The family comprises air-breathing marine limpets found globally on intertidal rocky shores except for polar regions (Hubendick 1946, Hodgson 1999). There are 205 nominal species described worldwide (White & Dayrat 2012, Dayrat *et al*. 2014). Together with other related limpet-like taxa such as *Williamia* and *Trimusculus*, they were traditionally regarded as basal in the pulmonates (White & Dayrat 2012), but more recently were considered to be more closely related to saccoglossans (Zapata *et al.* 2014, Bouchet *et al.* 2017).

Siphonariids graze on micro- and macroalgae (Hodgson 1999). Many of them maintain home scars to which they return after foraging (Branch 1981, Hodgson 1999). They have a limpet-like shape and their shell lengths vary from roughly 40 mm in *Siphonaria gigas* from Panama to the tiny 4-mm shells of *Siphonaria compressa*, which is restricted to beds of the estuarine seagrass *Zostera capensis* in South Africa (Angel *et al.* 2006, Wilson *et al*. 2009).

In southern Africa their geographic ranges cover a broad spectrum. At one extreme lies *Siphonaria compressa*, which is extremely localised, currently regarded as being restricted to only two lagoons in the Western Cape, and considered highly endangered and the rarest endemic marine mollusc in South Africa (Herbert 1998, 1999). At the opposite extreme, the wide-spread *Siphonaria capensis* is found along the entire southern African coastline from Angola to Mozambique (Branch *et al.* 2016). Three other South African species are relatively widespread: *Siphonaria serrata*, *S. concinna* and *Siphonaria oculus*. Four species that are restricted to the coasts of eastern South Africa and southern Mozambique (*Siphonaria nigerrima, S. tenuicostulata*, *S. anneae* and *S. dayi*) have recently been conflated into one (Teske *et al*. 2007) and given the name *Siphonaria nigerrima*.

Even from the early descriptions of *Siphonaria* species, there has been much confusion regarding application of names (Hubendick 1946). Anatomists often assigned names that differ from those employed today, so that multiple synonyms exist. Naming species on the grounds of shell features alone has compounded the problems, which continue to this day. Added to this, high levels of phenotypic plasticity (especially in shell shape and colour) sometimes makes identification based on shells alone problematic, leading to much debate globally about the identification and delineation of the species in the genus, and their relative phylogenetic relationships (Klussmann-Kolb *et al.* 2008, Jörger *et al*. 2010, White & Dayrat 2012).

In short, the family Siphonariidae and especially the genus *Siphonaria* have proved taxonomically difficult, and relationships amongst the species and with other groups have never been adequately resolved. The same situation existed for the patellid

limpets in the 1980s, but this has since been addressed by means of phylogenetic analyses (Koufopanou *et al*. 1999, Ridgway *et al*. 1998), although doubts still exist about the finer resolution of genera (Nakano & Ozawa, 2007).

Central to my work, Dayrat *et al.* (2014) elucidated the identification and phylogeny of *Siphonaria* species in the Indo-West Pacific. Their analysis did not, however, include the South African species, and one of the main aims of this thesis is to redress this and cast the southern African species in a global perspective.

More specifically, to date no genetic analysis has been done on *S. compressa*. This species was for many years known only from one population in Langebaan Lagoon in which it is known to have undergone radical population collapses and expansions. Reasons for its rarity and constrained habitat (Angel *et al*. 2006, Siebert & Branch 2007) and how its narrow salinity tolerance restricts it to estuaries with a narrow range of salinities (Wilson *et al*. 2009) have been described. A second living population was recently discovered in Knysna Lagoon (Allanson & Herbert 2005), 450 km to the south-east. Phylogeographical questions, such as how these two populations are related and how all the southern African species are positioned in the global phylogeny, have yet to be answered.

In addition to providing clarity about the relationships among the species, my thesis also explores ecological correlates such as mode of larval development and variations in diversity related to latitude.

Siphonariids as a model for investigation

Members of the Siphonariidae have been popular research targets over many years because of their small size, easily accessible intertidal habitat, abundance and high species diversity (see Hodgson 1999 for a review). As such they have proved themselves to be versatile and fascinating research animals.

Southern African members of *Siphonaria* have provided material for researchers asking questions on a wide range of topics, including systematics and distribution (Allanson 1958, Teske *et al.* 2007), the structure of egg-ribbons (Pal & Hodgson 2003), reproduction and growth (Allanson & Msizi 2010, Allanson & Fearon 2013), homing behaviour (Branch & Cherry 1985), taxonomy (Chambers & McQuaid 1994a), sperm morphology (Hodgson *et al.* 1991), mode of larval development (Chambers & McQuaid 1994b), genetic diversity and systematics (Chambers *et al.* 1996, 1998), chemical defences (McQuaid *et al*. 1999), salinity tolerance (Allanson 1958, Wilson *et al*. 2009), marine biogeography (Teske *et al*. 2011), tolerance to sand inundation and hypoxia (Marshall & McQuaid 1989, 1992, 1993), respiration (Kankondi *et al.* 2018) and ecology (McGlathery *et al.* 2012).

Most species of siphonariids are unpalatable and experience little natural predation (Hodgson 1999, Pinchuck *et al*. 2015). The compounds providing this protection have also been the subject of a number of studies, including exploratory research for novel bioactive molecules (Rovirosa & San-Martín 2006, Avila & Angulo-Preckler 2020). Elsewhere in the world, other species have been the subject of similarly

broad ranging investigations such as siphonariid digestion and feeding anatomy (Landro *et al.* 2019, Murty *et al*. 2013), morphometric diversity (Tablado & Gappa 2001) and microsatellite analysis (Wood & Gardner, 2010).

Species identification: a molecular approach

Historically, species identification of siphonariids relied mainly on anatomical characters, shell form and morphometric analyses (Rohlf 1990, Adams *et al*. 2013). However, over the last few decades the rise of ribonucleic acid sequencing and analytical techniques, and increases in computing speed and statistical power, have added a whole suite of new methods to many areas of biology (Hudson 2008, Schuster 2008). DNA sequencing is today well recognized a reliable method of species identification (Bucklin *et al.* 2011), with the identification of many species by DNA barcoding alone, or other DNA analysis (e.g. nuclear DNA), to support morphometric, anatomical and behavioural data (Yeates *et al.* 2011, Goulding & Dayrat 2016). As such, species delimitation using sequences has become central to DNA taxonomy (Zhang *et al.* 2013) and can accelerate biodiversity studies especially where morphological or ecological data are scarce (Monaghan *et al.* 2009).

Sequence data are also a powerful tool for estimating phylogenetic relationships among taxa (Simon *et al.* 1994, Ballard & Rand 2005) and this approach is a fundamental component of the relatively new discipline of phylogeography (Knowles 2009) which explores the spatial and temporal dimensions of genetic lineages, especially within and among closely related species (Avise 2009, Goulding *et al.* 2018, 2021) and has been employed to track recent changes in the abundances of genetic lineages for periods exceeding a century (Melroy & Cohen 2021). Molecular methodology has thus become a part of integrative taxonomy and is an accepted method of species identification and taxonomic resolution, complementing more traditional morphometric and morpho-anatomical data. The aim of this thesis is to use molecular techniques to help resolve issues regarding the identity, taxonomy, phylogeny and biogeography of the southern African siphonariids in particular.

Visual identification of siphonariid individuals is often problematic due to extreme intraspecific variability in shell colour and shape, coupled with morphological similarity among species and the relative uniformity of shell form within the genus (White & Dayrat 2012). South African *Siphonaria* have, since the very first formal descriptions, been the subject of taxonomic controversy and, to this day, confusion exists about the identity of species in South Africa. There is also ignorance about their relative relationships in terms of the global phylogeny of the genus and, thus, internationally the genus and affinities of species continue to be surrounded by uncertainty. This provides the rationale for this thesis.

Gene trees and species trees

Here, I briefly address a number of criticisms that have been raised concerning the molecular approach. A major criticism regarding the use of DNA sequences is that the

history of a single gene may not necessarily be the history of a species, and may even obscure true relationships among species. This concern has, however, largely been dealt with both in the literature and in comparative and simulation studies. According to Templeton (2001, p779), "a gene tree is an evolutionary reconstruction of the genealogical history of the genetic variation found in a sample of … DNA regions" subject to little or no recombination. It is thus a tool to probe the interface between intra- and interspecific evolution where speciation occurs.

Single gene sequences must be seen to generate *phylogenetically informative* gene trees (haplotype trees in some of the cases I explore) rather than the actual true historical evolutionary species trees. The two types of tree, gene and species trees, do differ. However, the gene trees generated by mitochondrial DNA sequences are a valid preliminary step towards exploring species diversification. In addition, the higher taxonomic relationships above the level of species are generally thought to be the same for both trees (Zhang *et al.* 2013).

My work utilised two genes, COI and 16S, albeit at a single locus, as they are both in the mitochondrial genome and are thus effectively inherited together and result in one gene tree.

Using only mitochondrial DNA

Another area of contention is the use of mitochondrial DNA exclusively in phylogenetic studies without the corroboration of nuclear sequences. However, it has been argued that there is a distinction between general *pattern* and quantifiable *process* in molecular studies, with mitochondrial DNA being a sensitive indicator of pattern and ideal for estimating geographical population structure, phylogeographic patterns and geographical and taxonomic species limits of recently evolved groups. This is the realm of phylogeography which deals with the spatial arrangements of genetic lineages especially within and among closely related species (Avise 2009). The rapid evolution of mitochondrial genes provides a high number of haplotypes and thus sufficient variation from which to draw inferences about gene flow and genetic structure, and for constructing geographically structured phylogenetic trees (Zink & Barrowclough 2008). More traditional population genetics approaches to quantifying gene flow and estimating other demographic variables such as population size, population growth and divergence times and resolving phylogeny above species level would need to complement mitochondrial DNA with nuclear sequences (Zink & Barrowclough 2008).

In defence of the use of mitochondrial DNA for species delimitation, Hebert *et al.* (2003) proposed DNA barcoding by means of sequence divergence thresholds in a single gene, COI, as a way of identifying species, discriminating among closely related species and discovering cryptic species. However, researchers caution against the use of DNA identification based on the nearest match and using an arbitrary set threshold of sequence divergence to identify taxa (Monaghan *et al*. 2009). As the relevant threshold will be different among different organisms, a suggestion is to rather establish a baseline for a taxonomic group using some well-characterized species and use this to make inferences about species limits (Davison *et al*. 2009). Unfortunately, barcoding

has not been successful for all animal groups, as the intraspecific variation in COI is high and can overlap with interspecific variation (Yu *et al.* 2016). For example, stylommatophoran land snails can have up to 30% divergence of mitochondrial DNA within species (Davison *et al.* 2009). Despite the above, Dupuis *et al.* (2012) found that mitochondrial DNA has roughly similar success rates in species delimitation of closely related species as that of other markers, and increased geographic or population sampling did not significantly affect success – although they do recommend the use of multiple markers. Yu *et al.* (2016, p1336) found that for the distinction of molluscan species in particular, "a single mitochondrial gene would be fully competent".

Subsequent to the development of barcoding, new molecular approaches to single-locus species delimitation are constantly being devised (Dellicour & Flot 2015), as are other methods of clarifying species-level biological diversity (Carstens *et al.* 2013). ABDG barcode gap detection (Lefebure *et al.* 2006; Puillandre *et al.* 2012), single and multiple threshold Generalised mixed Yule-coalescent (GMYC) (Pons *et al.* 2006; Monaghan *et al.* 2009; Fujisawa & Barraclough 2013) and Bayesian GMYC (Reid & Carstens 2012) are three of the most popular methods that use a single locus.

It might seem rather limited to be dealing with only two genes in this thesis, especially now in the days of next generation sequencing and other high through-put genomics where it is almost routine to sequence the whole mitogenome and entire genomes can be sequenced. However, the cost of research and the validity of investing time and money in a project are always concerns. Furthermore, as can be seen from the above and the results that emerge in this thesis, my results strongly suggest the existence of distinct and undescribed species, on grounds of complete absence of shared haplotypes among populations. As such, this can serve as an exploratory step promoting further research. The use of COI and 16S is further justified in that a substantial number of *Siphonaria* species have been sequenced for these genes, and the information is documented in GenBank, permitting comparisons among a large number of species.

Species concept

A final consideration to be addressed when using molecular sequences to identify species is to establish the species concept that should be adopted. There are many different species definitions, each one associated with certain restrictions and specific implications for the type of research proposed (Frankham *et al*. 2012). In an attempt to circumvent the ongoing controversy, De Queiroz (2007) argues that there is a difference between species conceptualisation and species delimitation. He suggests a unified species concept based on the idea that all alternative species concepts agree that the primary defining property of a species is that it is a separately evolving metapopulation lineage (an ancestor-descendant series). This becomes the only necessary property, and all secondary species criteria or defining properties (reproductive isolation, reciprocal monophyly, etc.) are classified as operational criteria, differing lines of evidence, or steps on the path to the final species. These steps will occur at different times and in a different order for different species during the course of lineage separation, divergence and speciation. Species conceptualisation is thus confirmed when separately evolving

lineages are identified, while species delimitation would require one or more of the operational criteria to be satisfied. This fits in with the current integrative taxonomic framework (Goulding & Dayrat 2016), which requires more data in the form of morphological, geographical, ecological and behavioural characters, together with molecular sequence data from different genes, to support a delimitation (Puillandre *et al.* 2012).

An example is illustrative. In birds, 97% of avian subspecies do not have a mitochondrial DNA population genetic structure typical of a distinct evolutionary unit, i.e., they do not exhibit reciprocal monophyly as would be required by the phylogenetic species concept (Zink 2004). They do, however, show geographically linked morphological differences. The incongruence between their mitochondrial gene trees and their subspecific limits is as a result of the different rates of morphological and molecular evolution.

This condition, in which rapid morphological divergence is not accompanied by genetic differentiation, is sometimes referred to as anti-cryptic speciation (Bickford *et al.* 2007). It is possible that some *Siphonaria* species fall into this category, in which apparently strong distinguishing morphological features are not always reflected genetically as, for example, the morphological distinction between putative *S. dayi* versus *S. nigerrima* (Teske *et al.* 2007). Given this intermediate phase in which genetic markers appear to lag behind faster-evolving phenotypic characters, it is suggested that subspecies in continental birds should thus be seen as an incipient phase of speciation before monophyly (Zink 2004). This may also be the case for some groups of *Siphonaria*. The comparison between bird sub-species and mollusc lineages and species is of course not perfect. However, I adopt this unified species concept in this thesis, with species conceptualisation being defined as identification of evolutionarily independent lineages.

To summarize, in this thesis I adopt the unified species concept (de Queiroz 2007) and an evolutionary biological approach and, utilising the mitochondrial genes 16S ribosomal ribonucleic acid (16S rRNA) and cytochrome oxidase subunit 1 (COI), I explore questions about the identity, taxonomy, phylogenetic relationships and biogeography of this group of limpets based on the principle of molecular evolution: that the degree of similarity between gene sequences reflects the strength of the evolutionary relationship among individuals. As such, the main thrust of this thesis is based on my genetic analyses; descriptions of species recognised genetically are supported by a consideration of distinguishing shell features, but I have not undertaken any anatomical or radular studies.

Thesis Outline

After this general introduction and outline of the thesis (Chapter 1), in Chapter 2 I resolve the identification of those South African species that are currently ambiguous, and their relationships with each other. In particular, I address firstly whether or not *Siphonaria oculus* and *S. concinna* can be regarded as distinct species. Secondly, I consider a complex that once embraced four species – *S. tenuicostulata*, *S. anneae*, *S. dayi*

and *S. nigerrima* - which have been merged under the name *S. nigerrima* as being conspecific (Teske *et al*. 2007). I expand the analysis of this complex by adding material from both farther North and farther South in southeastern Africa, and review the complex once more.

In Chapter 3 I examine more closely the critically endangered and rarest endemic marine mollusc in South Africa, the lagoonal *S. compressa*. The main questions addressed are whether the two extant populations that have been recorded are indeed the same species, how they are related and their genetic variability.

In Chapter 4 I use the same mitochondrial DNA markers to expand on the current global molecular phylogeny of the genus (Dayrat *et al.* 2014) – from which all South African species are currently absent. In addition, the phylogeny I developed allows various ecological and biogeographic aspects of the genus to be analysed and discussed. The final chapter then provides a synthesis of this work.

To conclude, the layout of this thesis is as follows:
Chapter 1: *Introduction* – overview of the background of the thesis and the problems to be addressed.
Chapter 2: *Elucidation of Siphonaria species on the East Coast of Southern Africa* – resolution of the specific status of some southern African siphonariids within a phylogenetic framework.
Chapter 3: *Siphonaria compressa, South Africa's rarest endemic mollusc: one species or two?* Here, I focus on the range-restricted and endangered *S. compressa* of the Western Cape region of South Africa, to determine if the two extant populations are a single species or not.
Chapter 4: *Integrating southern African species into a world phylogeny of Siphonaria species, and an analysis of biogeographic patterns in the genus* – adds to the current global phylogeny of *Siphonaria* by expanding the number of species included and, in particular, adding all South African species.
Chapter 5 *Synthesis and Conclusion* – a concluding and synoptic chapter that draws together the central messages emerging from the thesis.

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Chapter 2
Elucidation of *Siphonaria* species on the East Coast of Southern Africa.

ABSTRACT

Frequent changes of names and synonymizing of species has created uncertainty about the number of species of *Siphonaria* that exists in South Africa, and the names that should be applied to them. Here, I focus on (1) resolving the status of clades within a complex of species currently recognised as *S. carbo* and (2) the distinction between *Siphonaria concinna* and *S. oculus*.

Research by Teske *et al.* (2007), using mitochondrial and nuclear sequence data on *Siphonaria nigerrima*, *S. anneae*, and *S. tenuicostulata*, failed to discern distinct monophyletic lineages, thus indicating that these species, together with *S. dayi*, are different colour morphs of a single species. Teske *et al.* (2007) therefore merged these four species under the name *S. nigerrima*, thereby reducing the number of South African *Siphonaria* species to seven. This is in contrast to the previous systematic revision by Chambers & McQuaid (1994), which considered *Siphonaria nigerrima*, *S. anneae*, *S. tenuicostulata* and *S. dayi* to be separate species, and identified nine species in South Africa.

An added complication is that the name *S. nigerrima* has been synonymised with *S. carbo* by various authors, including Hubendick (1946), Allanson (1958) and Kilburn and Rippey (1982), and the latter name is recognised as valid in a range of data bases. Chambers and McQuaid (1994), however, took the view that this name cannot be validly applied to South African material. Resolution of the number of species in this complex that are present in South Africa, and the appropriate name(s) that should be applied, is therefore needed.

A separate issue is that the distinction between *S. concinna* and *S. oculus* has become blurred in the literature, with only the former being identified by Teske *et al.* (2011).

Using genetic analyses based on COI and 16S ribosomal RNA genes, I examined the relationships among members of the '*S. carbo*' complex and the distinction between *S. oculus* and *S. concinna*. In both cases I incorporated sequences available in GenBank. In addition, I examined the type material of *S. carbo* to explore the validity of employing that name for South African representatives of the complex.

My work confirms that *S. nigerrima* and *S. dayi* comprise a single species, to be merged under the name *S. nigerrima* because of its priority as stipulated by the ICZN (International Commission on Zoological Nomenclature), and because examination of the type material of *S. carbo* indicates that it is unlikely that that species is present in South Africa. However, I also show that within the '*S. nigerrima* complex' there exist four or possibly even five clades. Clade A (sampled at Tofo, Mozambique) and Clade B (sampled at Paraiso de Chidenguele, Mozambique) appear to be distinguishable as separate species, with Clade A being an undescribed species, whereas the name *S. tenuicostulata* may be applicable to Clade B. Clade C comprises the bulk of the

specimens examined, and I attribute the name *S. nigerrima* to this clade. Clade D contains material from Durban and Clade E from Mkambati, and it is conceivable that the name *S. anneae* can be attached to this pair of clades, although I cannot with certainty draw that conclusion. A clear genetic cline emerged between Clade A in the North, and Clade E in the South.

With respect to *S. oculus* and *S. concinna*, my molecular analyses and a consideration of shell features provide evidence that the two are distinguishable and valid species.

In summary, (1) I confirm the conflation of two east coast species, *S. nigerrima* and *S. dayi* into a single species under the name *S. nigerrima*, (2) propose that there are more species of *Siphonaria* in South Africa than recognised by the revision of Teske *et al*. (2007), with my molecular data suggesting the presence of additional species identifiable as genetically distinct clades within the '*S. nigerrima* complex', two of which may be assignable to the names *S. anneae* and *S. tenuicostulata*, (3) formally propose that the name *S. carbo* not be applied to South African material based on an examination of the type material of *S. carbo* and multiple shells of *S. nigerrima* and the conclusion that the two are different species and that the name *S. nigerrima* be employed in its stead, and (4) clarify the relationships between *S. concinna* and *S. oculus*, showing that the two are distinct, as evidenced from both genetic and shell characteristics.

I also assess the validity of the names assigned to other putative South African species listed in databases, including GenBank, and provide corrections for some of these names.

2.1 INTRODUCTION

South African members of the false limpet genus *Siphonaria* Sowerby, 1824 have a muddled taxonomic history, with multiple and repeated mergers and splits of species, with some simply overlooked in the course of revisions. Multiple names and synonymies have been applied by various collectors over the years leading to perplexity about how many species there are in South Africa and what names should be assigned to them.

Initial work was morphological in nature, with collectors in the 19th and 20th century in some cases generating new names for species that had already been described. In the last decade, despite the welcome addition of molecular techniques for identification, problems have continued to plague identification within the genus. Coupled with a lag in updating public data bases, this has resulted in a confusing situation.

A brief systematic history of southern African Siphonaria

Table 2.1 overviews the taxonomy of *Siphonaria* in South Africa, outlining the treatment of names and synonymies of South African species from before the first major revision by Hubendick (1946) to the present.

The first five columns are reproduced in part from Chambers & McQuaid (1994) in which they describe the history of the taxonomic changes in South Africa up to 1994.

The next four columns have been added for this thesis to update the taxonomy and show the current position. It is clear that there has consistently been indecision regarding the status and correct application of names for a number of species. The taxonomic status prior to 1946, reflecting the species described by various researchers between 1807 and 1944, is shown in Table 2.1, Column 1.

The 1946 Hubendick monograph on the systematics of the Siphonariidae provided the first formal treatment of the South African species. Considering all species then known world-wide, he defined ten 'sectia' in the genus *Siphonaria* divided between two subgenera (*Liriola* and *Siphonaria*) based on the organisation of the distal genitalia, the veins from the gill and kidney and the shell. However, the term 'sectia' has no formal status in the International Code for Zoological Nomenclature (Anonymous 1999, Dubois 2011), and few have adopted this level of division for the genus. *Liriola* is absent from South Africa, but three of Hubedick's sectia of *Siphonaria* occur on southern African shores: *Pachysiphonaria, Patellopsis* and *Siphonaria*, and are sometimes treated as subgenera. Hubendick (1946) recognized and redescribed 13 South African species (Table 2.1, Column 2, with the sectia in parentheses). Hubendick omitted *S. anneae*, possibly because he was not aware of Tomlin's description of this species, as both Tomlin and Hubendick published relatively closely together, in 1944 and 1946, respectively.

In the first major revision of southern African species after Hubendick, Allanson (1958), using shell characteristics and the morphology of the reproductive and respiratory organs, found only five of Hubendick's South African species to be valid and declared the rest to be synonyms or doubtful. He resurrected the name *S. deflexa* for *S. concinna* (although this was later overturned), adopted *S. anneae* Tomlin 1944, and described two new species and one variety: *Siphonaria compressa*, *Siphonaria dayi* and *Siphonaria aspera* var. *pallida* (Table 2.1, third column).

Table 2.1 Taxonomic nomenclature applied to southern African *Siphonaria* pre-1946 to present. First five columns reproduced from Chambers and McQuaid (1994 Table 1), updated in the next four columns to reflect the status of species at the start of the thesis. Subgenera employed by Hubendick (1946) and others are shown in parentheses. Blank cells indicate the author did not address the taxon. The right hand column summarises the findings of my thesis.

Prior to 1946	**Hubendick, 1946, with sectia or subgenera in brackets**	**Allanson, 1958**	**Kilburn and Rippey, 1982**	**Chambers and McQuaid, 1994**	**Teske *et al*., 2007**	**Branch *et al*., 2016**	**Catalogue of Life catalogueoflife.org** Accessed 1/12/2021	**MolluscaBase molluscabase. org** Accessed 18/12/2021	**This thesis**
S. deflexa **Helbling 1779**	Synonym of *S. concinna*	*S. deflexa*					Synonym of *S. concinna*	*nomen dubium*	*S. concinna*
S. concinna **Sowerby, 1824**	*S. concinna* (*Siphonaria*)	*S. deflexa* (*Patellopsis*) (Helbling, 1779)	*S. concinna*	*S. concinna*		*S. concinna*	*S. concinna*	*S. concinna*	***S. concinna***
S. concinna. var albofasciata **Krauss, 1848**	S. albofasciata (*Siphonaria*)	Synonym of *S. deflexa*	Synonym of *S. concinna*	Synonym of *S. concinna*			Synonym of *S. concinna*	Synonym of *S. concinna*	Synonym of *S. concinna*
S. cyaneomaculata **Sowerby, 1906**	*S. cyaneomaculata* (*Siphonaria*)	Synonym of *S. deflexa*	Synonym of *S. concinna*				Synonym of *S. concinna*	Synonym of *S. concinna*	Synonym of *S. concinna*
S. concinna **var. *adjacens*** **Turton, 1932**	*S. adjacens* (*Siphonaria*)	Synonym of *S. deflexa*		Synonym of *S. concinna*			Synonym of *S. concinna*	Synonym of *S. concinna*	Synonym of *S. concinna*
S. variabilis **Krauss, 1848**							Synonym of *S. concinna*	Synonym of *S. concinna*	Synonym of *S. concinna*
S. carbo **Hanley, 1858**	*S. carbo* (*Patellopsis*)	*S. carbo* (*Patellopsis*)	*S. carbo*	Incorrect name for Sn African species			*S. carbo*	*S. carbo*	Name not applicable to any Sn African species
S. nigerrima **Smith, 1903**	Synonym of *S. carbo*		Synonym of *S. carbo*	*S. nigerrima*	*S. nigerrima*	*S. nigerrima*	Synonym of *S. carbo*	*S. carbo*	***S. nigerrima***
									Siphonaria **sp. nov. Mkambati**
									Siphonaria **sp. nov. Tofo**
S. tenuicostulata **Smith, 1903**	*S. tenuicostulata* (*Patellopsis*)	Synonym of *S. anneae*?	*S. tenuicostulata*	*S. tenuicostulata*	Synonym of *S. nigerrima*	Synonym of *S. nigerrima*	Synonym of *S. carbo*	Synonym of *S. carbo*	***S. tenuicostulata***
S. anneae **Tomlin, 1944**		*S. anneae* (*Patellopsis*)		*S. anneae*	Synonym of *S. nigerrima*	Synonym of *S. nigerrima*	Synonym of *S. carbo*	Synonym of *S. carbo*	***S. anneae***
		S. dayi **(*Patellopsis*) Allanson, 1959**		*S. dayi*	Synonym of *S. nigerrima*	Synonym of *S. nigerrima*	*S. dayi*	*S. dayi*	***S. nigerrima***
S. oculus **Krauss, 1848**	*S. oculus* (*Siphonaria*)	*S. oculus* (*Patellopsis*)	*S. oculus*	*S. oculus*		*S. oculus*	*S. oculus*	*S. oculus*	***S. oculus***
S. becki **Turton, 1932**	*S. becki* (*Siphonaria*)	synonym of *S. oculus*					Not listed	*taxon inquirendum*	Synonym of *S. oculus*
S. capensis **Quoy & Gaimard, 1833**	*S. capensis* (*Patellopsis*)	*S. capensis* (*Patellopsis*)	*S. capensis*	*S. capensis*		*S. capensis*	*S. capensis*	*S. capensis*	***S. capensis***
S. kowiensis **Turton, 1932**	*S. kowiensis* (*Pachysiphonaria*)	Synonym of *S. capensis*?					Not listed	*taxon inquirendum*	*nomen dubium*
S. serrata **Fischer, 1807**	*S. aspera* (*Siphonaria*) (Krauss, 1848)	*S. aspera* (*Siphonaria*)	*S. aspera*	*S. serrata*		*S. serrata*	*S. serrata*	*S. serrata*	***S. serrata***
		S. aspera **var. *pallida*** **(*Siphonaria*) Allanson 1959**	Synonym of *S. aspera*				Not listed	*S. pallida* – *taxon inquirendum*	Synonym of *S. serrata*
S. natalensis **Krauss, 1848**	*S. natalensis* (*Siphonaria*)	Synonym of *S. aspera*	Synonym of *S. aspera*	Synonym of *S. serrata*			Synonym of *S. serrata*	Synonym of *S. serrata*	Synonym of *S. serrata*
S. parcicostata **Deshayes, 1863**	*S. parcicostata* (*Siphonaria*)	Sn African records doubtful					*S. parcicostata*	*S. parcicostata*	Unconfirmed; probably absent from Sn Africa
		S. compressa **(*Sacculosiphonaria*) Allanson, 1959**	*S. compressa*	*S. compressa*		*S. compressa*		*S. compressa*	***S. compressa***
									Siphonaria **sp. nov. Knysna**

Kilburn and Rippey (1982) acknowledged seven species including *Siphonaria compressa* but did not recognise the other two additions made by Allanson (Table 2.1, fourth column). They preferred the use of *S. tenuicostulata* to Allanson's use of *S. anneae*.

Chambers and McQuaid (1994) produced the next major review of the group's taxonomy in South Africa, using shell characteristics and larval development; they concluded that there were nine valid species in the region. They argued that the name *S. carbo* Hanley, 1858 could not validly be applied to any South African species as they believed it was unlikely that the type specimen came from South Africa, and proposed that the name for the South African species should be changed to *S. nigerrima* Smith, 1903.

In further work, and using polyacrylamide gel electrophoresis (PAGE) of total soluble proteins, Chambers *et al.* (1996) examined seven species of *Siphonaria* and found that there were lower levels of genetic variability within direct-developing species (*S. anneae*, *S. nigerrima*, *S. serrata* and *S. tenuicostulata*) than in planktonic-developing species (*S. concinna*, *S. capensis* and *S. oculus*). Protein absorbance profiles were similar for all species, leaving their higher systematic relationships unclear although, when data from all species were compared in a single dendrogram, individuals of the species with direct development nested together but were distinct from each other (Chambers *et al*. 1996).

Chambers *et al.* (1998) then addressed the systematic relationships of nine species of South African *Siphonaria* and three foreign species using Random Amplified Polymorphic DNA fingerprinting (RAPD). They confirmed their previous conclusions on the systematic relationships for the nine species based on morphology and PAGE. However, in contrast to their previous study, the within-population variability was now greatest for the direct developing species. They found a close relationship between *S. concinna* and *S. oculus*, with *S. capensis* being allied but distinct. *Siphonaria anneae* and *S. tenuicostulata* were clear sympatric sister taxa and nested together with *S. nigerrima* and *S. dayi*. However, for one of the primer sets, "*S. nigerrima* and *S. dayi* failed to nest as coherent groups" (Chambers *et al.* 1998, p54). They considered that two species not recognised by Kilburn and Rippey (1982) were supported by genetic data: *S. anneae* and *S. dayi*. By including *S. tenuicostulata,* they brought the number of east-coast species up to four and the total of southern African species to nine (Table 2.1, Column 5). Richmond (2011, p252) has listed *S. tenuicostulata* as occurring in East Africa as far as the Arabian Gulf, but notes there are "at least three other similar species" there.

There have since been additional genetic analyses that have altered the picture once again. In contrast to the Chambers and McQuaid (1994) systematic revision, subsequent research by Teske *et al.* (2007), using mitochondrial and nuclear sequence data of *S. nigerrima*, *S. anneae*, *S. tenuicostulata* and *S. dayi*, was unable to recover distinct monophyletic lineages. They did detect two closely

related lineages in this complex, which grouped on the basis of geography (subtropical and tropical) rather than morphology, with specimens collected north and south of Cape St Lucia forming distinct monophyletic clusters. The combined genetic diversity of the four species was found to be lower than that of other South African species and fell within the range predicted for a single species.

Teske *et al.* (2007) therefore proposed that all four species (*Siphonaria nigerrima*, *S. tenuicostulata*, *S. anneae* and, *S. dayi*) are colour morphs of *S. nigerrima*, and that Cape St Lucia can be confirmed as a phylogeographic break in their genetic continuity. The specific name *nigerrima* was chosen in accordance with current nomenclatural rules (i.e., chronologically by year, and sequence within paper) based on the description by Smith (1903). Thus, the number of South African species was reduced to six (Table 2.1, Column 6).

In other phylogeographic work by Teske *et al.* (2011), two distinct evolutionary lineages of *S. concinna* were identified. However, the same research failed to identify *S. oculus* among the individuals sampled, raising questions about the validity of this species.

Finally, Branch *et al.* (2016) followed Teske *et al.* (2007) in considering the three species as synonyms of *S. nigerrima* and recognized six *Siphonaria* species (Table 2.1, Column 7). This is the current state of southern African siphonariid systematics.

As on-line databases and the public's access to them has become ever more popular, it is the duty of scientists to ensure their contents are correct. The public databases Catalogue of Life (catalogueoflife.org) and MolluscaBase (molluscabase.org) (Table 2.1, Column 8), require resolution of some of the names they apply to *Siphonaria* species.

Firstly, based on the research of Teske *et al.* (2007), databases have synonymised *S. nigerrima*, *S. anneae*, and *S. tenuicostulata* but the name has reverted back to *S. carbo* Hanley, 1858 instead of the suggested *S. nigerrima* (MolluscaBase.org). This is problematic as the occurrence of *S. carbo* in southern Africa was specifically mentioned by Chambers and McQuaid (1994) as being unlikely. In addition, *S. dayi* has remained listed in databases as a valid species even though Teske *et al.* (2007) suggested that it should be synonymised with *S. nigerrima*, together with *S. anneae* and *S. tenuicostulata*.

In other databases such as Global Biodiversity Information Facility (GBIF), Encyclopaedia of Life (eol.org) and Gastropods.com the photographs and the corresponding names of the species and their distributions are often incorrect. Central to this thesis is the resolution of these issues for southern African species, to which I return in the Discussion section of this chapter.

As an aside, E.T. (Tex) Reid, an amateur conchologist, spent an enormous amount of time and effort in the 1990s on the morphological and anatomical characterisation of east-coast African members of the genus and identified numerous putative species. By mid-1995 he had drafted eight manuscripts

containing a wealth of information on most of the South African *Siphonaria,* all in an advanced stage of preparation, which – sadly – were never published. He performed hundreds of dissections, studied thousands of *Siphonaria* shells, and on the basis of detailed morphology of the gut, reproductive organs and number and structure of radular teeth (using scanning electron microscopy) identified individuals that he believed to be members of up to ten new species, while also confirming the other species identified by Chambers and McQuaid (1994). In the early 1980s he had already posited the currently accepted idea of a biogeographic break at the St Lucia area (Sink *et al.* 2005; Porter *et al.* 2017) as he believed that northern Natal was where there was a "change of species" and that southern Mozambique/northern Natal was an ecological dividing point.

In one of his manuscripts (dated 1996), Reid suggested a sub-genus named *Afrosiphonaria* with non-planktotrophic development, occurring on the east African coast to the Red Sea and the Gulf of Iran, in small isolated pockets. These species have similar anatomy and shell characteristics and the following characteristics made them distinct from all other *Siphonaria*: non-planktotrophic development and the form of the spermathecal duct, seminal vesicle, atrial complex and epiphallic complex. His sub-genus *Afrosiphonaria* included the following species: *S. anneae, S. asghar, S. dayi, S. kowiensis, S. nigerrima, S. tenuicostulata, S. crenata*. He considered that there were seven undescribed species.

To my reading, his work was thorough, detailed and impressive, and featured diagrams of the gill complex and variation in kidney form, the epiphallic gland complex, the seminal vesicles, the bursa copulatrix and spermatophores, the coelom (showing the enlarged hind intestine), the atrial complex and the reproductive gland complex. Additional characters he used to differentiate among species were the number of shell ribs, specific shell markings, the robust porcellaneousness (sic) or thinness and fragility of shell, spatula colour and shell dimension ratios.

He called for a major taxonomic review of the group and was well aware of the inadequacy of shell characteristics alone as identifying features, as they may vary more intraspecifically than interspecifically. He corresponded with other researchers well known in the field and exchanged specimens with South African, European and British institutions.

Although his work was neither published nor subjected to peer review, I feel that many of his observations and conclusions are especially relevant to the questions posed in this chapter: whether the reduction of four species into one, *S. carbo*, is valid and whether there may be more than the currently accepted six species in the region. I will thus include and consider his work in the Discussion.

In the Discussion I will return to Table 2.1 and refer to the final right-hand column, which shows, firstly, the species names that I consider are valid on grounds of supporting evidence I supply in this chapter and, secondly, new clades that are likely to represent unrecognised species that were identified

during my research.

In summary, this chapter probes the identification of certain species of the southern African members of the genus. The analysis has four foci. Firstly, I consider the four putative species *S. nigerrima*, *S. tenuicostulata*, *S. anneae* and *S. dayi*, currently subsumed under the name *S. carbo*, and explore (a) whether or not this conflation is justified and (b) if the name *S. carbo* can validly be applied to this complex. Secondly, I address whether or not there is a difference between *S. concinna* and *S. oculus*, which needs to be resolved as a result of Teske *et al.* (2011) treating them as a single species, and I clarify whether they constitute distinct species or should be merged. Thirdly, I correct the identification of some of the South African species registered under incorrect names on GenBank. Fourthly, and finally, I resolve in the Discussion the validity of some of the earlier names that still exist in databases.

2.2 MATERIALS AND METHODS

Sample collection

Between 2005 and 2011 specimens were collected from intertidal locations around South Africa and Mozambique, and immediately preserved in 90-98% ethanol. For the purpose of this chapter, I focus on the material for species whose identity required resolution, i.e., those provisionally identified morphologically as *S. concinna, S. oculus*, *S. nigerrima*, *S. tenuicostulata*, *S. anneae* and *S. dayi* based on the nomenclature of Chambers and McQuaid (1994) and confirmed by RAPD analysis (Chambers *et al.* 1998) (see Table 2.1).

Samples were provisionally identified by me and independently confirmed by GM Branch of the University of Cape Town. Specimens that did not clearly conform to existing descriptions or appeared to be novel were labelled with a 'Q'. Voucher specimens from the Iziko South African Museum and the KwaZulu-Natal Museum were obtained and comparisons and confirmations were made. The accession numbers for the KwaZulu-Natal Museum specimens are as follows: *S. anneae,* NMSA V52 (Umhloti), V51 (Umhlanga Rocks); *S. capensis,* NMSA V46; *S. compressa,* NMSA V45; *S. concinna*, NMSA V50; *S. dayi*, NMSA V47; *S. nigerrima*, V49; *S. oculus*, NMSA V53; *S. serrata*, V55 (Waterloo Bay), V56 (Kommetjie), V57 (Umhloti); *S. tenuicostulata,* NMSA V48 (Chambers & McQuaid 1994). The Iziko Museum specimens were: *S. dayi* Allanson paratype; *S. anneae* (*olim S. tenuicostulata*) Tomlin, KOS 22F, D246; *S. carbo* (*olim S. nigerrima*) (Hanley), D243; *S. aspera* Krauss E208A, F364; *S. aspera* var *pallida* Allanson LB175C; *S. deflexa* Helbling, D89, F332; *S. oculus*, Kr D90; *S. compressa* Allanson paratype, LB221A; *S. capensis* Q and G., B156, D22 (Allanson 1958). In addition, high-resolution images of the type specimen of *S. carbo* were provided by the Natural History Museum, London, to allow comparison with specimens putatively of the same species collected in South Africa.

Figure 2.1 shows the currently accepted distribution of the species and clades dealt with in this chapter, while Appendix 2.1 lists the samples collected, and Appendix 2.2 specifies the accession numbers of additional relevant sequences obtained from GenBank (Benson *et al.* 2013). Combining the sequences from GenBank with those of my own analyses, 101 sequences were compared; 43 for the *S. carbo/nigerrima* complex, and 58 for *S. oculus/concinna*.

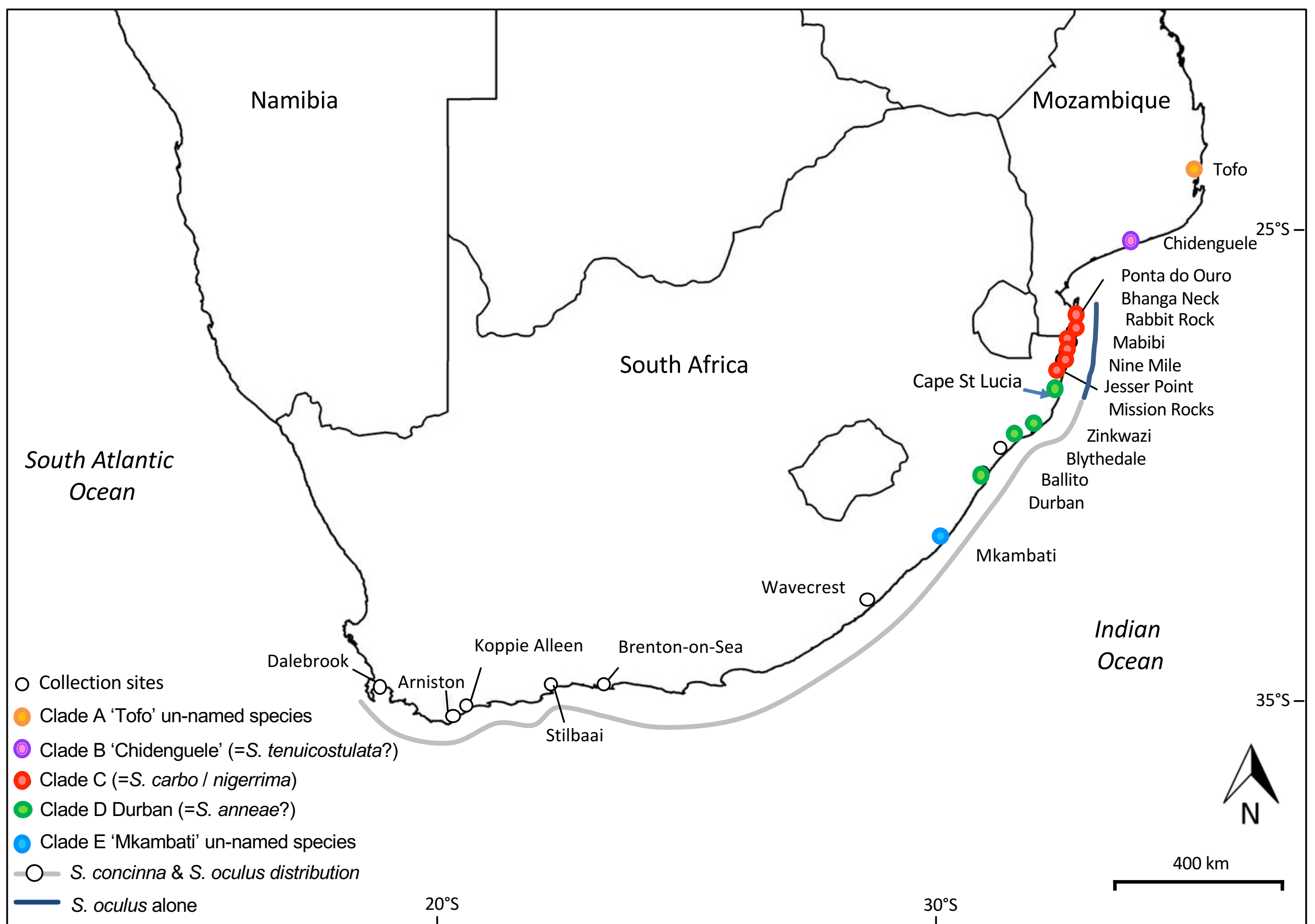

Figure 2.1 Geographic ranges of *Siphonaria concinna* and *S. oculus* (after Branch *et al.* 2016), the positions of sites where samples were collected, and the clades I recognized in the *S. nigerrima* complex.

Laboratory procedures

(A) DNA extraction
All extraction was performed using a sterile procedure to prevent cross-contamination. A section of ethanol-preserved foot muscle roughly 2-3mm in diameter (10-20mg) was excised with a scalpel, rinsed in clean 90% ethanol to remove any attached material (sand, gut contents, faeces) and genomic DNA extracted using a modified CTAB (hexadecyltrimethylammoniumbromide) DNA extraction protocol (Doyle & Doyle 1987, Saghai-Maroof *et al.* 1984).

Lastly, the DNA was suspended in 100µl Gentra PuregeneTM DNA Hydration Solution or TE (tris(hydroxymethyl)aminomethane (Tris)-Ethylenediaminetetraacetic acid (EDTA)) buffer and left overnight at room temperature and subsequently stored at 4°C (for immediate use) or -20°C (for long-term storage). The presence of DNA was checked on a 0.8% agarose/TBE (Tris/Borate/EDTA) gel.

(B) Choice of markers
Eukaryote mitochondrial DNA contains genes encoding the protein subunits of the respiratory chain complexes responsible for oxidative phosphorylation and the formation of adenosine triphosphate (ATP), ribosomal ribonucleic acid (RNA) and a set of twenty-two transfer RNAs (Avise 2009). The genes are an essential part of cellular energy and protein manufacture and are thus strongly conserved.

Individual organisms are homoplasmic, i.e. all their mitochondrial DNA has an identical nucleotide sequence and thus one genotype or haplotype only. Each individual will differ from another only by the accumulation of mutations since their female ancestor was last shared (Avise 2009).

Different genes have different rates of evolution (for example 16S evolves more slowly than COI (Lefebure *et al.* 2006)) and are subject to different constraints, so they can be used for different purposes. Because there is little or no genetic recombination, the mitochondrial genes are all linked in a single, non-recombining, circular chromosome so that the whole DNA sequence is one clonally inherited, single haplotype regarded as a single locus only. This makes it especially useful for studying population history.

Their relatively rapid evolution and subsequent high nucleotide sequence variation, generating high intraspecific polymorphism, makes them popular in studies of both population genetics and phylogenetic relationships (Moritz *et al.* 1987) and they have been "the mainstay of intraspecific phylogeography and of systematics" (Sunnucks 2000, p 201) where the haplotype sequences are used to estimate the matrilineal histories of individuals and populations (Avise 2009).

In addition, their relative abundance, and the absence of introns (spacer regions) and indels (inserted or deleted lengths of sequence) between the single copies of genes make them technically easy to manipulate (Simon *et al.* 1994).

COI genes are relatively easily sequenced with universal primers (Folmer *et al.* 1994).

Due to the smaller effective population size of the maternally inherited mitochondrial DNA, haplotypes coalesce or become monophyletic faster than nuclear markers (Moore 1995). This implies new species should become distinct in their haplotype phylogenies long before they do so in their nuclear markers (Wiens & Penkrot 2002). This is one of the many reasons the COI gene has also been the gene of choice for the barcoding of organisms and other methods of species delimitation using mitochondrial sequence variation (Zink & Barrowclough 2008).

The non-protein-coding mitochondrial ribosomal RNA (rRNA) genes code for a single strand of RNA which is folded to create secondary structures, which – combined with proteins – create a tertiary structure or scaffold involved in protein assembly (Simon et al. 1994). Although they are non-protein coding, they need to conserve functionally important positions where specific proteins bind for the scaffolding process, and thus typically comprise highly conserved sequence blocks that provide attachment sites for other proteins, interspersed with highly variable sections that are subject to weaker functional constraints.

I used the 16S ribosomal RNA gene together with the mitochondrial cytochrome *c* oxidase subunit I (COI) gene, which codes for one of the most highly conserved protein-coding genes in animal mitochondria.

Appendix 2.3 shows the primers employed.

(C) DNA amplification and sequencing

16S reactions*:* The 16S 25 µl polymerase chain reactions (PCR) contained 2.5 µl 10x buffer, 2.5 µl 25 mM magnesium chloride ($MgCl_2$), 2.5 µl 1 mM deoxyribonucleotide triphosphate (dNTPs), 1.25 µl 10 µM) forward (16Sar) primer, 1.25 µl 10 µM reverse (16Sb) primer, 0.25 µl Super Therm DNA Polymerase (JMR-801)(Super-Therm Taq enzyme), 2 µl template DNA extract and 12.75 µl purified water. For each PCR run one blank control (i.e. template DNA was replaced by water) was included.

The 16S PCR profile was as follows: 94°C for 3 minutes; 35 cycles of 94°C for 30 seconds, 48°C for 45 seconds, 72°C for 1 minute; 72°C for 5 minutes. The PCR product was electrophoresed, visualised with GoldviewTM Nucleic Acid Stain (SBS Genetech) on a 1% agarose/(Tris/Borate/EDTA)(TBE) gel (at 100 Volts), the band excised and DNA purified from the gel slice with a Qiagen QIAquick® Gel Extraction Kit. The clean-up was checked by gel electrophoresis on a 2% agarose/TBE gel.

COI reactions*:* The COI 25 µl PCR contained 2.5 µl 10x buffer, 3µl 25 mM $MgCl_2$, 2.5 µl 1 mM dNTPs, 1.25 µl 10 µM forward (LCOI490) primer, 1.25 µl 10 µM reverse (HCO2198) primer, 0.25 µl Super-Therm PolymeraseTaq enzyme, 2 µl template DNA and 12.25 µl purified water. For each PCR run one blank control

(i.e. template DNA was replaced by water) was included. Note the additional 0.5µl $MgCl_2$ necessary to optimise the amplification. The COI PCR profile was as follows: 94°C for 2 minutes; 35 cycles of 94°C for 30 seconds, 46°C for 30 seconds, 72°C for 45 seconds; 72°C for 10 minutes. For sequences difficult to amplify DNA template volume was doubled from 2µl to 4µl and annealing times were varied between 46°C and 50°C for COI.

Sequencing was provided by Central Analytical Facilities at University of Stellenbosch, South Africa, using Big Dye terminator chemistry on an ABI3100 automatic sequencer. A number of sequencing reactions were performed with the reverse primer to check accuracy of the forward primer sequence. Base calls were confirmed by aligning both strands.

Sequence analysis

(A) Sequence assembly

The sequences were assembled in CodonCode Aligner version 3.7 (Codon Code Corp, http://www.codoncode.com), which automatically reverse-complements the sequences thus ensuring the correct uniform direction of the sequences in the traces so that they are all aligned in the 5'-3' direction.

The peak quality at all polymorphic sites (where two peaks were present in a trace) was checked visually and all peaks at that position for all individuals sequenced were compared. The base representing the largest peak was always chosen whether or not this corresponded with the other individuals at this site or not. This was done to ensure that all sites were used as data by the analytical program's algorithm so as to maintain the site as an informative character and to prevent it from being treated as an uncertain character or classified as missing information. The existence of the secondary peak implies there is a second haplotype present, however weakly resolved. Double peaks in traces were more common in the 16S sequences.

(B) Multiple sequence alignment

The ends of all the sequences were manually trimmed. Once assembled, the 16S sequences were aligned with the multiple sequence alignment program Muscle (Edgar 2004), with default settings via Jalview 2.8 (Waterhouse *et al.* 2009), and the alignment refined by eye in Jalview and Bioedit (Hall 1999).

Assembled COI sequences were translated into protein with RevTrans 2.0 (Wernersson and Pedersen 2003) using the invertebrate mitochondrial genetic code (number 5) and with the 'no start codon' option chosen. RevTrans 2.0 translates the sequences into amino acids, aligns them in the multiple sequence alignment program MAFFT (Katoh *et al.* 2002), and backtranslates the longest open reading frames from the correct frame. This ensures the correct frame of three is selected and the resultant amino acids are correct for the protein. The retrieved sequences were again aligned with Muscle, with default settings via

Jalview 2.8 and the alignment refined by eye in Jalview and Bioedit.

The 16S and the corresponding COI sequences from the same individual were combined with package ape v4.0 (Analysis of Phylogenetics and Evolution) (Paradis *et al*. 2004) for R v3.3.2 (R Core Team, 2016) in Rstudio v1.0.136 (RStudio Team, 2015).

Additional relevant COI sequences were obtained from GenBank (Appendix 2.2) and subjected to the same assembly and translation process. They were added to my COI dataset to make an additional expanded COI dataset.

The study produced a total of 86 new sequences, all of which will be submitted to GenBank. The shells of specimens will be deposited with the KwaZulu-Natal Museum, Pietermaritzburg, South Africa where the Chambers & McQuaid (1994) samples are currently stored.

(C) Tree building

Substitution models were evaluated in Topali v2.5 (Milne *et al.* 2009) and computed remotely via The James Hutton Institute, Scotland, which offers the models available in the programs MrBayes and PhyML (Guindon *et al.* 2010). Hierarchical likelihood ratio tests, Akaike information criterion (AIC) and Bayesian information criterion (BIC) were generated for each possible model. Models with the lowest BIC scores are considered to describe the most suitable substitution model for the dataset (Bofkin & Goldman 2006).

Rate variation was addressed by partitioning the data into coding (COI) and non-coding (16S) portions and generating specific models for each. In addition, a model was generated for each of the three codon positions in the coding COI dataset by choosing the codon option.

HKY+G (with the lowest BIC of 3542.28) was chosen as the 16S alignment model and HKY+I (lowest BIC of 4775.86), was the chosen model for the COI dataset. These were selected as both PhyML and MrBayes generated the lowest BIC scores for these substitution models for these data. These models were then included for each respective gene when preparing the command block for the combined 16S+COI dataset in MrBayes. For the COI + GenBank dataset, the codon model option was selected from the MrBayes substitution models (first position K80+G, second position F81 and third position HKY+I) as it provided the lowest BIC.

Initial exploratory neighbour joining, parsimony and maximum likelihood trees (not shown) were constructed using ape v4.0 and pegas v0.9 (Population and Evolutionary Genetics Analysis System) (Paradis, 2010) in Rstudio v1.0.136 with R v3.3.2. The subsequent choice of outgroup was based on the results of these trees. Final Bayesian ultrametric trees (in which branch lengths correspond with either relative or absolute time) were obtained from cladograms including the posterior probabilities for each split using MrBayes v3.2.3 and computed remotely via Cipres Science Gateway v3.3 (Miller *et al.* 2010).

MrBayes uses Bayesian analysis and probabilistic models to construct phylogenetic trees. As the posterior probability of phylogenetic trees and the other parameters of the model cannot be determined analytically, a stochastic algorithm, Metropolis-Coupled Markov Chain Monte Carlo (MCMCMC), estimates distributions by drawing samples from the posterior probability distribution, over the parameter state space, given the data, under the chosen model of evolution. The posterior probability distribution is made up of the likelihood combined with the prior (the prior is the probability distribution over parameter space prior to seeing the data, i.e. the assumptions about probabilities of parameter values before the data are analysed).For the tree this is typically "flat" i.e. all possible trees are equally likely so the initial probability is 1/total possible trees.

Successful MCMCMC inference depends on the run performance of the Markov chain. Convergence and stationarity of the runs and adequate mixing of the chains will give the correct posterior probabilities (clade credibility) for each split or taxa bipartition in the resultant cladogram or tree. Hence it is important to monitor the process continuously and repeatedly manipulate settings to achieve a credible result.

To ensure that the Bayesian analysis had converged and stationarity had been achieved, the following parameters were optimised: run length, sampling frequency (and thus sample size and number of trees used in generating the final tree), model choice (coding, non-coding and three codon model for protein-coding COI), temperature heating parameter (to improve mixing), chain number and burn-in percentage.

Trace plots (generation vs log probability of observing the data) were observed in Tracer v1.6.0 (Rambaut *et al.* 2014) to monitor the MCMCMC run and the efficiency of the mixing as the MCMCMC algorithm sampled the parameters. Good mixing behaviour is shown by a dense, "hairy caterpillar", trace with no large-scale fluctuations. Other tree parameters and the posterior probability of the topology of the tree were visualised with AWTY (Are We There Yet) (Nylander *et al.* 2008).

Optimisation was achieved when the following convergence diagnostics achieved suggested levels: average Standard Deviation of Split Frequency (aSDSF) tended to zero, acceptance rates of parameter changes fell between 20% and 50%, chain swapping between adjacent chains was between 10% and 70%, Estimated Sample Size (ESS) (of independent samples) was greater than 200, Potential Scale Reduction Factor (PSRF) approached 1 as runs converged and autocorrelation time (ACT) plots were smooth and unimodal. Once conditions were optimised, runs were repeated with the same conditions to ensure consistency. Resultant trees were visualised in Figtree v1.4.0 (Rambaut 2016). Appendix 2.4 provides details on the settings and convergence diagnostics for multiple MrBayes runs.

(D) Haplotype construction

Parsimony haplotype diagrams were constructed in RStudio v1.0.136 using R v3.3.2 and packages ape v4.0 and pegas v0.9.

(E) Summary statistics and distance analysis

Sequence statistics and measures of sequence variation and genetic population diversity for COI were calculated in RStudio v1.0.136 using the following packages in R v3.3.2: ape v4.0 and pegas v0.9.

Nucleotide diversity (π), a measure of the degree of polymorphism or genetic variation within a population at the level of nucleotides, was calculated by counting the average number of nucleotide differences between any two DNA sequences chosen randomly from the sample (average pairwise nucleotide difference) (Nei & Li, 1979). This pairwise distance matrix between sequences was constructed using the default Kimura 2-parameter model (Kimura 1980). It was also calculated for other species of *Siphonaria* to allow comparisons of genetic diversity across species. There were no sites with missing or ambiguous data.

The number of segregating sites (positions at which there are polymorphisms) is used in calculating another measure of nucleotide polymorphism, θ, which is subject to the requirements of Kimura's (1969) infinite-sites mutation model. This model requires that the number of nucleotide sites is large enough such that each mutation will occur at a site that has not mutated before; that the populations are in equilibrium between mutation and random genetic drift; and that the mutations are neutral.

The neutral theory of molecular evolution predicts that change will accumulate gradually at a constant rate and that most changes are selectively neutral (Kimura 1968). This accumulation of genetic change can be used to estimate differentiation within and between populations with the passage of time. While strict neutrality is not always the case, many statistical tests assume it as the null hypothesis. π and θ should be the same under the neutral theory of molecular evolution.

To test the neutral mutation hypothesis, Tajima's D neutral evolution statistic was calculated with p as the p-value assuming that D follows a normal distribution (Tajima 1989). Tajima's D test puts a number on the difference between the estimate of θ calculated from the number of segregating sites and the average pair-wise sequence divergence. Negative values are interpreted as a sign of positive selection or a rapidly growing population while positive D values indicates balancing selection, or a population reducing in size. The closer the value of D is to zero, the more randomly or 'neutrally' the sequences are evolving.

Distance analysis was performed in MEGA (Kumar *et al.* 2018) to show genetic distances among the species I recognize and percentage differences among the individuals of each species (Tamura *et al.* 2004).

Shell characteristics

All specimens used for genetic analysis were photographed, together with relevant voucher and type specimens, and representative shells collected in the same areas as specimens employed for genetic analyses. Dimensions, textures and colour patterns were recorded to distinguish morphotypes.

2.3 RESULTS

The following sequence datasets were developed: (1) a 16S dataset (43 sequences by 461 base pairs), (2) a COI dataset (43 sequences x 669 base pairs) and (3) a concatenated 16S+COI dataset (43 sequences x 1130 base pairs). (4) An expanded COI dataset of 104 sequences x 672 base pairs was generated by pooling my COI sequences with those of an additional 61 relevant sequences obtained from GenBank. These sequences were mainly of *S. concinna* and *S. oculus* (to confirm my identification of them as two species) while nine were *S. nigerrima* from areas close to where I sampled (Appendix 2.2).

Bayesian trees

A Bayesian tree showing my combined 16S and COI dataset, with *S. capensis* as the outgroup and with branch labels indicating probabilities (Figure 2.2), demonstrates two findings. Firstly, it indicates that *S. oculus* and *S. concinna* are sister taxa that are genetically distinct from each other. Secondly, within the *S nigerrima* complex, it reveals a clade of at least four (and possibly five) subclades, three of which form a polytomy. The largest group (Clade C, from Bhanga Neck and Rabbit Rock) comprises specimens that had *a priori* been identified at *S. nigerrima.* It was also possible to identify four clades that include potential new species (labelled with a 'Q') that were distinguished in the field on the basis of shell morphology as being different from currently recognised taxa. These site-specific 'queries' or 'unknowns' proved to be genetically different from Clade C: Clades D and E (Durban and Mkambati, respectively), Clade A (Tofo) and Clade B (Chidenguele).

The COI Bayesian tree for my COI sequences plus GenBank sequences, with *S. capensis* once again as the outgroup (Figures 2.3 A, B), corroborates the distinction between *S. oculus* and *S. concinna,* and also showed that multiple GenBank sequences identified as *S. concinna* and even one named *S. capensis* are in fact *S. oculus* (Figure 2.3A).

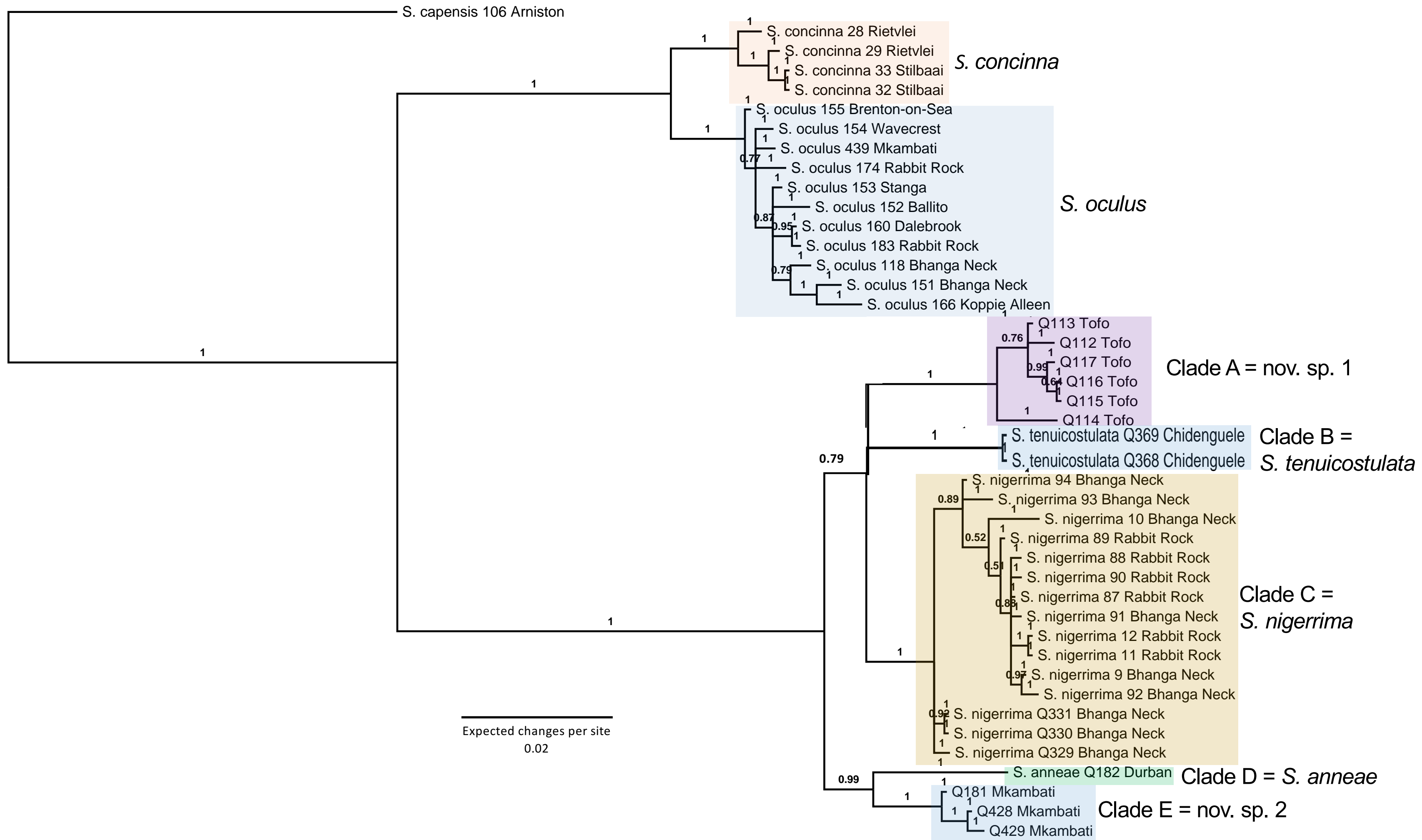

Figure 2.2 Bayesian tree with *S. capensis* as the outgroup, combining my 16S and COI sequences, with branch labels indicating probabilities. Sample names for all individuals as they appear on the tree were those given when the specimens were initially identified prior to DNA analysis.

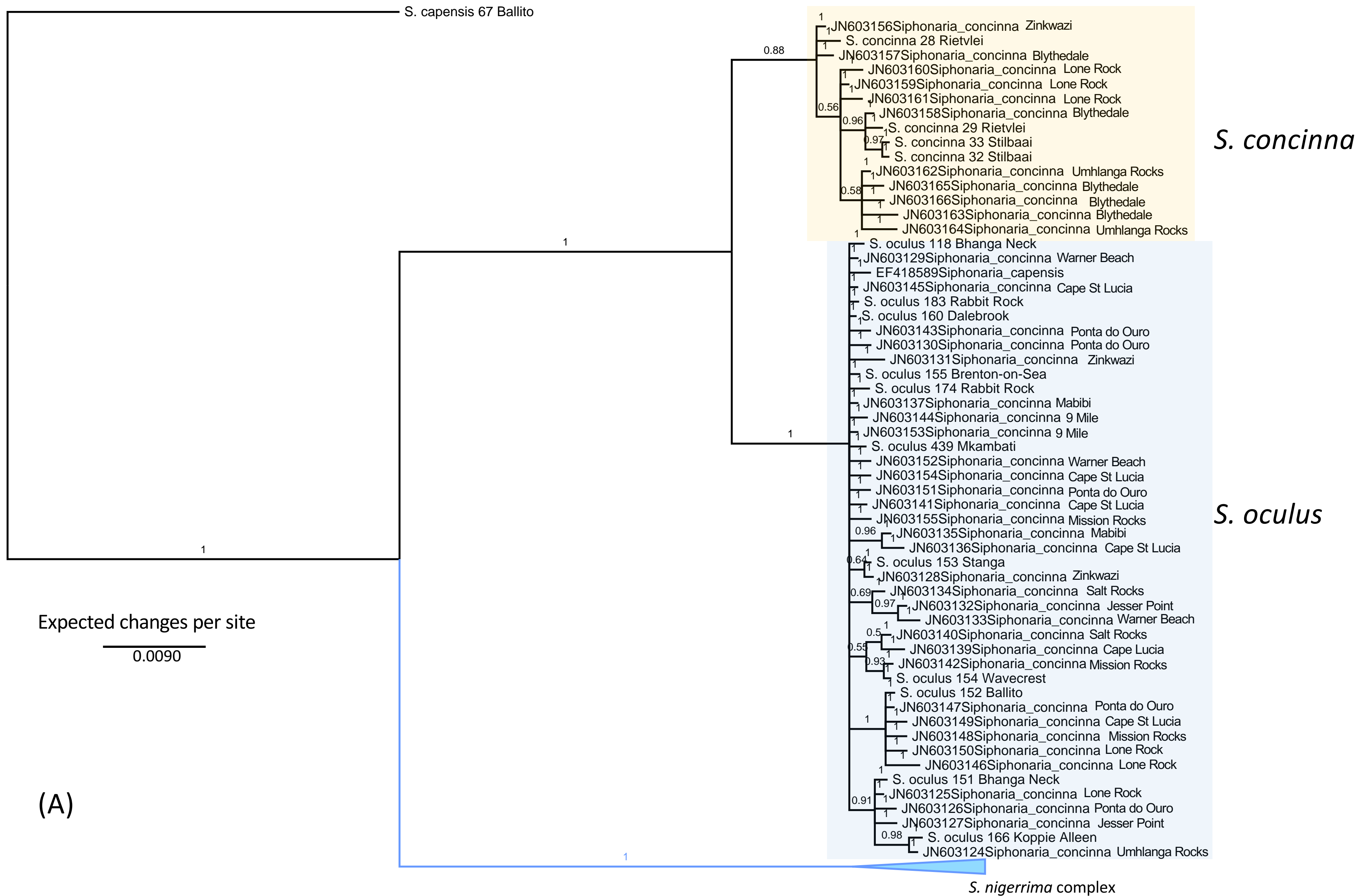

Figure 2.3 (A) See caption on next page.

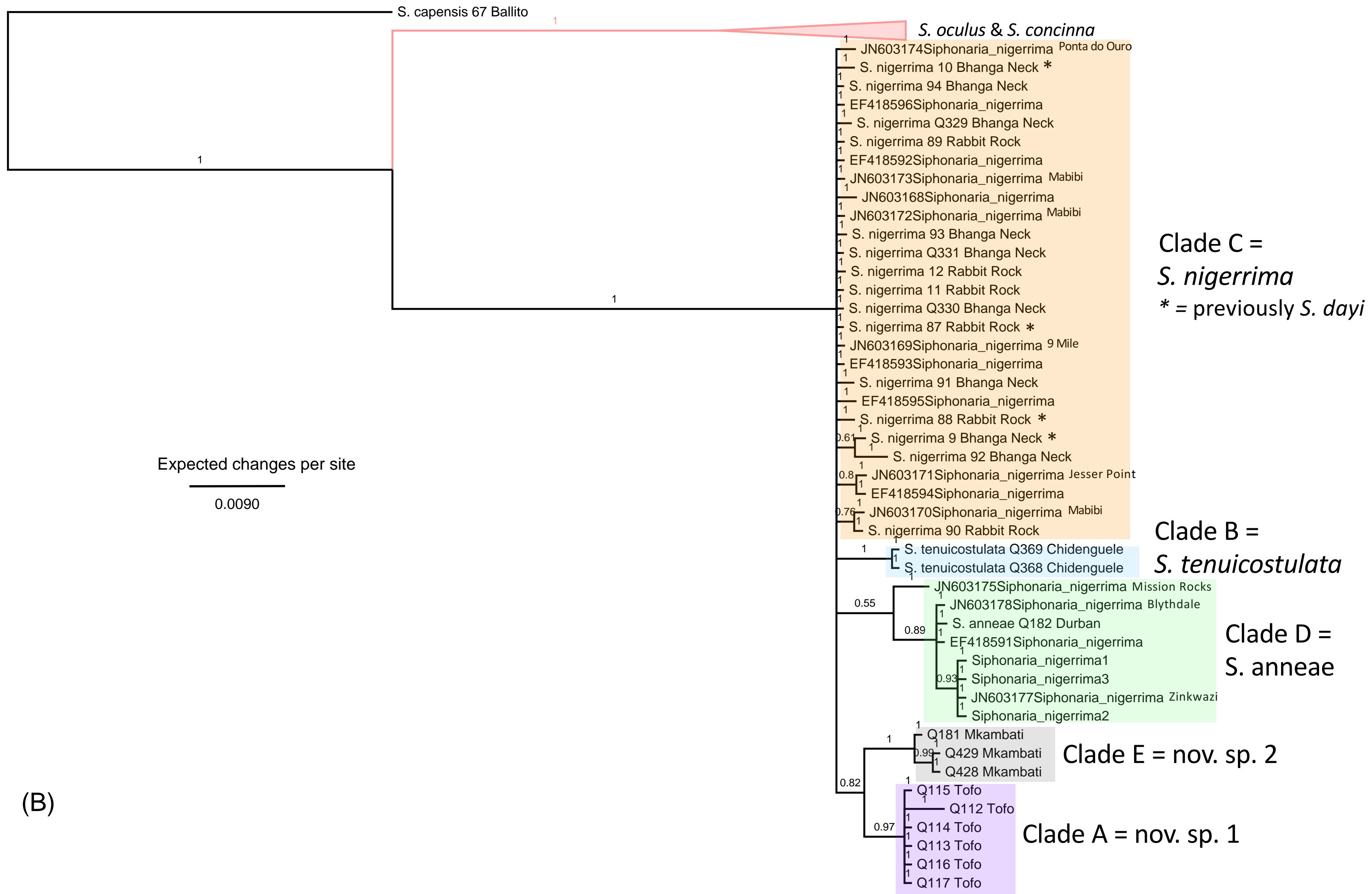

Figure 2.3 The Bayesian tree for COI sequences only, including both my COI sequences and GenBank sequences, with *S. capensis* once again as the outgroup. The tree has been divided into (A) the *S. concinna* and *S. oculus* portion and (B) the '*Siphonaria nigerrima*' complex for ease of viewing.

Figure 2.3B again corroborates the result found for my concatenated dataset for the *S. nigerrima* complex (Figure 2.2), although the taxa were somewhat less well resolved for COI, and the monophyly of Clade C was not recovered. However, for Clade A (Tofo), Clade B (Chidenguele), and Clade E (Mkambati), the site-specific sequences still emerged together in the same geographic localities and retained their clade status in the polytomy, thus corresponding to the concatenated analysis. Clade D expanded from a single individual into a clade with the addition of several GenBank sequences (although the posterior probability of 0.55 was quite low), and was no longer closely associated with Mkambati. However, it is notable that my sample from Durban clustered together with the GenBank samples from Zinkwazi, Blythedale and Mission Rocks, thus forming a group that lies geographically to the south of Clade C (Fig. 2.1), and that the junction between the two fell at a recently recognised biogeographic break at Cape St Lucia (Teske *et al*. 2007) . Another feature is that Clade C incorporated both dark-shelled forms that would previously have been termed *S. nigerrima* or *S. carbo* in the narrower definition of that species, and pale forms that would have been termed *S. dayi*.

Mission Rocks does lie 16 km North of Cape St Lucia. However, the geographic break proposed at that locality by Teske *et al*. 2007 would extend over some distance and overlap can be expected between the two regions, rather than there being a precise point location.

Appendix 2.2 shows the current species names and GenBank accession numbers for the additional sequences obtained from GenBank that I used in my analysis. Figure 2.3B reveals that many of these have incorrect specific names in the GenBank database. In particular, a large proportion of samples (JN603124–JN603155) listed as *S. concinna* are in fact *S. oculus*; but there is also an instance of an *S. oculus* incorrectly named as *S. capensis* (EF418589). I have updated Appendix 2.2 to indicate the correct names.

Haplotypes

Figure 2.4A shows the fourteen 16S haplotypes in a haplotype parsimony network of 27 individuals for the *S. nigerrima* complex from 6 sites; Figure 2.4B the COI haplotype parsimony network with 18 haplotypes for the same 27 individuals; and Figure 2.4C the COI haplotype parsimony network for 36 individuals (26 haplotypes) from 13 sites, combining my COI sequences and those obtained from GenBank.

For the 16S gene (Fig. 2.4A), the haplotypes were almost all site-specific, with none shared other than between Bhanga Neck and Rabbit Rock, which, given their geographical proximity to each other, is not surprising. In a regional context, based both on similarity of haplotypes and their geographic positioning, five groups existed: two in the north at Tofo and Chidenguele, a large central cluster, and two in the south at Durban and Mkambati.

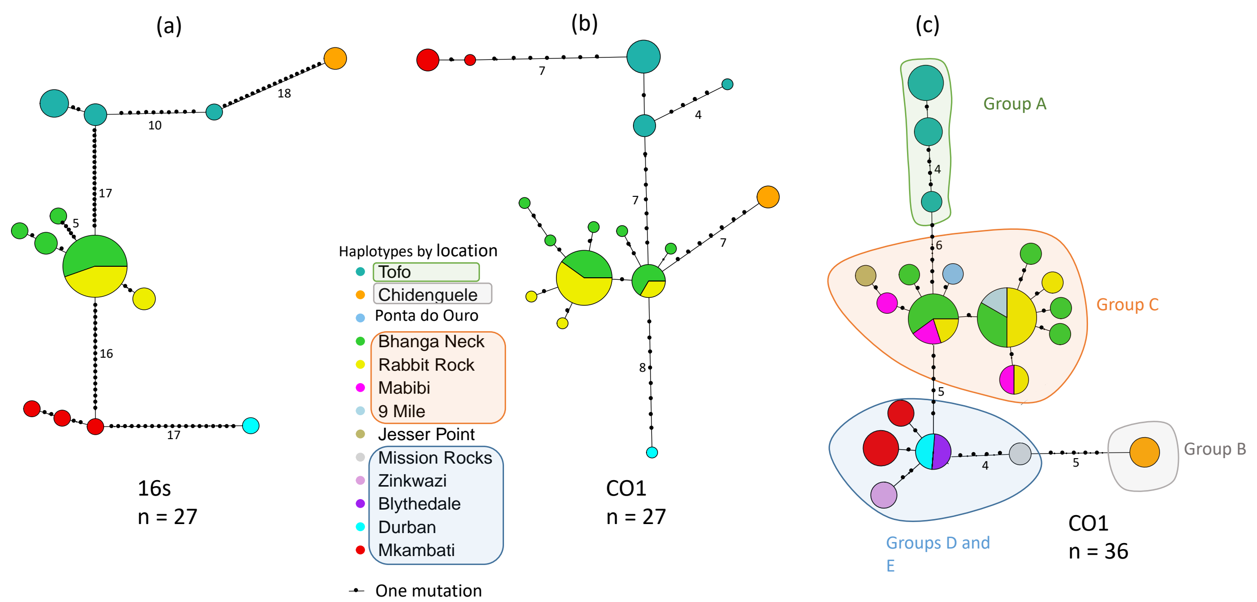

Figure 2.4 Haplotype parsimony networks for the '*Siphonaria nigerrima*' complex, for (A) 16S, (B) COI and (C) an extended COI dataset of individuals made up of my sequences (see Appendix 2.1) and sequences obtained from the GenBank (Appendix 2.2). Sites are listed from north to south. Small black dots indicate numbers of substitutions (possible unsampled haplotypes) and numbers are also provided when there are more than 3 substitutions. Each haplotype is colour-coded based on the site where it was collected.

The most informative haplotype network emerged from the COI haplotypes for the 36 individuals (Fig. 2.4C). A closely related cluster of haplotypes from six sites near the centre of the geographic range of the *S. nigerrima* complex occurred between Ponta do Ouro in the north and Jesser's Point in the south, which I have called Group C (corresponding to Clade C described below). To the south of this, Groups D and E (=Clades D and E) were found from Mkambati to Zinkwazi, possibly extending to Mission Rocks. To the North, the Tofo (=Clade A) and Chidenguele (=Clade B) haplotypes were each distinct. Groups C and B appear to be separated by the biogeographic break just north of St Lucia (Fig. 2.1).

The larger combined COI dataset results (Fig. 2.4C) corroborate those from my smaller COI dataset (Fig. 2.4B), with the exception that samples from Durban and Mkambati emerged as distinct from each other in my samples but were more closely allied in the combined data set. Fig. 2.1 shows the distribution of these distinct groups, demonstrating that a series exists from South to North in terms of the genetic composition of the material I incorporated in my analysis.

Sequence statistics and distance analysis

Table 2.2 summarises the summary statistics calculated for the species studied here. Results for *S. nigerrima* (incorporating *S. nigerrima* and putative *S. dayi* samples), *S. concinna* and *S. oculus* are provided for two sets of sequences: those that I sequenced, and a larger group that includes those retrieved from GenBank. In the case of *S. nigerrima*, inclusion of GenBank sequences more than doubled the number of individuals sampled (16 to 34), increased the number of sites at which these were collected (3 to 13), and affected haplotype number (9 to 17), nucleotide diversity (0.0036 to 0.0065) and segregating sites (15 to 19). The results for *S. concinna* reflect similar changes: number of individuals sampled (4 to 15), number of sites at which these were collected (9 to 11), haplotype number (10 to 32), nucleotide diversity (0.0085 to 0.0086) and segregating sites (21 to 27). *S. oculus,* however, did not show as much of a change as the other two species despite sampling across its range: number of individuals sampled (11 to 43), number of sites at which these were collected (2 to 6), haplotype number (3 to 14), nucleotide diversity (0.007 to 0.0088) and segregating sites (9 to 17).

Fewer samples were collected of members of the three clades from Tofo, Mkambati and Chidenguele. Samples from the individual sites of Tofo (n=6), Mkambati (n=3) and Chidenguele (n=2) had three, two and one haplotypes respectively with Tofo's nucleotide diversity 0.0029 and that of Mkambati 0.001. Tofo had five segregating sites while Mkambati had one and Chidenguele none.

Table 2.2 Sequence statistics and measures of sequence variation and genetic population diversity for COI; n = number of individuals, length = sequence length, H = number of haplotypes, theta = genetic divergence calculated from segregating sites.

species or site	n	number of sites	length	H	nucleotide diversity(π) (variance)	segregating (polymorphic) sites	theta (θ)	Tajima's D
S. nigerrima	16	3	650	9	0.0036 (0.0)	15	4.520	-1.874 p=0.061
S.nigerrima including Genbank	34	13	516	17	0.0065 (0.0)	19	4.647	-0.936 p=0.349
S. concinna	4	2	666	3	0.007 (0.0)	9	4.909	-0.492 p=0.623
S.concinna including Genbank	15	6	408	14	0.0088 (0.0)	17	5.228	-1.258 p=0.208
S. oculus	11	9	660	10	0.0085(0.0)	21	7.170	-1.007 p=0.314
S.oculus including Genbank	43	11	408	32	0.0086 (0.0)	27	6.240	-1.474 p=0.141
Tofo	6	1	666	3	0.0029 (0.0)	5	2.190	-0.655 p=0.512
Mkambati	3	1	669	2	0.001 (0.0)	1	0.667	
Chidenguele	2	1	669	1	0	0	0.000	
S. compressa	83	2	661	39	0.0103 (0.0)	36	7.214	-0.173 p=0.863
S. capensis	245	20	647	137	0.0201 (0.0001)	117	19.255	-0.995 p=0.319
S. serrata	18	18	664	12	0.0141 (0.0001)	37	10.757	-0.538 p=0.591

Results from *S. compressa* (n=83, 2 sites, H=39), *S. capensis* (n=245, 20 sites, H=137*)* and *S. serrata* (n=18, 18 sites, H=12) (all my own sequence data) were calculated for comparison: their nucleotide diversities (0.01; 0.02; 0.014) were an order of magnitude greater than those of *S. nigerrima, S. concinna* and *S. oculus*. Their segregating sites were 36, 117 and 37 respectively. Although none of the calculated values for Tajima's D showed significant departure from 0, all were negative, and that for *S. nigerrima* approached significance (p=0.06).

Percentage genetic distances (with standard errors) are given in Table 2.3. Within-group genetic distances (Table 2.3a) were always smaller than between-group distances and ranged from zero (Chidenguele, *S. tenuicostulata*) to 0.38% (Northern KZN, *S. nigerrima*). The between-group genetic distance (Table 2.3b) between Mkambati (furthest south) and Chidenguele (*S. tenuicostulata*) was the largest at 1.95% followed by Mkambati and *S. nigerrima* at 1.75%. The genetic between-group percentage was 1.72% for the Mozambican samples from Chidenguele (*S. tenuicostulata*) and Tofo. There was a 1.61% difference between Tofo and *S. nigerrima*. Between Tofo and *S. anneae* the

difference was 1.52% and between *S. tenuicostulata* and *S. nigerrima* it was 1.47%. There was 1.33% difference between *S. anneae* and *S. nigerrima*. The lowest percentage genetic difference was between *S. anneae* and Mkambati at 0.58%. The percentage differences among the individuals of each species are shown in Table 2.3c. *S. nigerrima* had the largest range, from 0% to 1.72%. The range for *S. anneae* was 0% to 1%, Tofo, 0% to 0.46%, Mkambati, 0% to 0.15% and *S. tenuicostulata* 0% (only two individuals).

Table 2.3 Estimates of average genetic evolutionary divergence over sequence pairs within-groups, (a), between-groups, (b), and among the individual sequences of each group, (c), as percentages. Standard error estimates are shown in blue or above the diagonal.

(a) Within Group

		SE
North KZN (*S. nigerrima*)	0.38%	0.0013
Central KZN (*S. anneae*)	0.34%	0.0017
Tofo (new)	0.29%	0.0015
Chidenguele (*S. tenuicostulata*)	0.00%	0.0000
Mkambati (new)	0.10%	0.0010

(b) Between Group

	North	Central	Tofo	Chid'le	Mk'bati
North KZN (*S. nigerrima*)		0.0043	0.0048	0.0046	0.0047
Central KZN (*S. anneae*)	1.33%		0.0049	0.0048	0.0021
Tofo (new)	1.61%	1.52%		0.0054	0.0043
Chidenguele (*S. tenuicostulata*)	1.47%	1.50%	1.72%		0.0055
Mkambati (new)	1.75%	0.58%	1.36%	1.95%	

(c) Per Group

Central KZN (*S. anneae*)	1	2	3	4	5	6	7	8
JN603175Siphonaria_nigerrima		0.0040	0.0047	0.0041	0.0047	0.0047	0.0040	0.0040
Q182_CO1_Dbn	0.79%		0.0020	0.0030	0.0020	0.0020	0.0000	0.0000
Siphonaria_nigerrima2_nigerrima	1.00%	0.19%		0.0020	0.0000	0.0000	0.0020	0.0020
JN603177Siphonaria_nigerrima	0.79%	0.39%	0.19%		0.0020	0.0020	0.0030	0.0030
Siphonaria_nigerrima_nigerrima	1.00%	0.19%	0.00%	0.19%		0.0000	0.0020	0.0020
Siphonaria_nigerima3_nigerrima	1.00%	0.19%	0.00%	0.19%	0.00%		0.0020	0.0020
EF418591sp	0.79%	0.00%	0.19%	0.39%	0.19%	0.19%		0.0000
JN603178Siphonaria_nigerrima	0.79%	0.00%	0.19%	0.39%	0.19%	0.19%	0.00%	

Mkambati (new)	1	2	3
Q181_CO1_Mkb		0.002	0.002
Q429_CO1_Mkb	0.15%		0.0000
Q428_CO1_Mkb	0.15%	0.00%	

Tofo (new)	1	2	3	4	5	6
Q115_CO1_Tofo		0.0015	0.0015	0.0000	0.0015	0.0029
Q114_Tofo	0.09%		0.0000	0.0015	0.0000	0.0033
Q113_Tofo	0.09%	0.00%		0.0015	0.0000	0.0033
Q116_Tofo	0.00%	0.09%	0.09%		0.0015	0.0029
Q117_CO1_Tofo	0.09%	0.00%	0.00%	0.09%		0.0033
Q112_CO1_Tofo	0.37%	0.46%	0.46%	0.37%	0.46%	

Chidenguele (*S. tenuicostulata*	1	2
Q369_XX		0.0000
Q368_CO1_XX	0.00%	

North KZN (*S. nigerrima*)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
EF418593sp		0.0000	0.0018	0.0017	0.0018	0.0019	0.0017	0.0029	0.0032	0.0017	0.0017	0.0019	0.0026	0.0019	0.0017	0.0026	0.0028	0.0017	0.0017	0.0024	0.0031	0.0000	0.0000	0.0000	0.0017	0.0000	0.0000
JN603169Siphonaria_nigerrima	0.00%		0.0020	0.0018	0.0020	0.0019	0.0019	0.0032	0.0032	0.0019	0.0019	0.0019	0.0026	0.0019	0.0019	0.0028	0.0028	0.0019	0.0019	0.0026	0.0027	0.0000	0.0000	0.0000	0.0019	0.0000	0.0000
ann091_CO1_BhN	0.18%	0.19%		0.0025	0.0022	0.0028	0.0021	0.0035	0.0039	0.0026	0.0022	0.0028	0.0034	0.0028	0.0039	0.0031	0.0035	0.0022	0.0021	0.0031	0.0031	0.0032	0.0048	0.0016	0.0051	0.0016	0.0032
EF418595sp	0.18%	0.20%	0.35%		0.0024	0.0026	0.0023	0.0033	0.0037	0.0024	0.0024	0.0027	0.0032	0.0026	0.0024	0.0031	0.0034	0.0024	0.0025	0.0029	0.0036	0.0017	0.0017	0.0017	0.0024	0.0017	0.0017
ten88_CO1_RR	0.18%	0.19%	0.31%	0.35%		0.0028	0.0022	0.0035	0.0039	0.0026	0.0022	0.0028	0.0034	0.0027	0.0027	0.0033	0.0034	0.0022	0.0022	0.0031	0.0031	0.0016	0.0016	0.0016	0.0022	0.0016	0.0016
JN603170Siphonaria_nigerrima	0.19%	0.19%	0.39%	0.39%	0.39%		0.0000	0.0026	0.0026	0.0027	0.0027	0.0027	0.0032	0.0028	0.0027	0.0035	0.0034	0.0027	0.0027	0.0032	0.0034	0.0019	0.0019	0.0019	0.0027	0.0019	0.0019
ann090_CO1_RR	0.18%	0.19%	0.30%	0.35%	0.31%	0.00%		0.0024	0.0026	0.0025	0.0021	0.0027	0.0032	0.0028	0.0040	0.0032	0.0034	0.0021	0.0021	0.0029	0.0031	0.0033	0.0049	0.0015	0.0052	0.0015	0.0033
EF418594sp	0.53%	0.59%	0.71%	0.71%	0.71%	0.39%	0.35%		0.0000	0.0024	0.0024	0.0026	0.0019	0.0039	0.0024	0.0031	0.0033	0.0024	0.0035	0.0017	0.0044	0.0029	0.0029	0.0029	0.0024	0.0029	0.0029
JN603171Siphonaria_nigerrima	0.59%	0.59%	0.79%	0.79%	0.79%	0.39%	0.39%	0.00%		0.0026	0.0026	0.0026	0.0019	0.0039	0.0026	0.0034	0.0033	0.0026	0.0039	0.0019	0.0044	0.0032	0.0032	0.0032	0.0026	0.0032	0.0032
EF418592sp	0.18%	0.19%	0.35%	0.35%	0.35%	0.39%	0.35%	0.35%	0.39%		0.0000	0.0000	0.0019	0.0027	0.0000	0.0018	0.0019	0.0000	0.0025	0.0017	0.0036	0.0017	0.0017	0.0017	0.0000	0.0017	0.0017
ann89_CO1_RR	0.18%	0.19%	0.30%	0.35%	0.31%	0.39%	0.30%	0.35%	0.39%	0.00%		0.0000	0.0019	0.0027	0.0032	0.0023	0.0019	0.0000	0.0022	0.0017	0.0030	0.0032	0.0048	0.0015	0.0045	0.0015	0.0032
JN603173Siphonaria_nigerrima	0.19%	0.19%	0.39%	0.39%	0.39%	0.39%	0.39%	0.39%	0.39%	0.00%	0.00%		0.0019	0.0027	0.0000	0.0020	0.0019	0.0000	0.0028	0.0019	0.0034	0.0019	0.0019	0.0019	0.0000	0.0019	0.0019
JN603172Siphonaria_nigerrima	0.39%	0.39%	0.59%	0.59%	0.59%	0.59%	0.59%	0.19%	0.19%	0.19%	0.19%	0.19%		0.0034	0.0019	0.0028	0.0027	0.0019	0.0035	0.0000	0.0039	0.0026	0.0026	0.0026	0.0019	0.0026	0.0026
JN603168Siphonaria_nigerrima	0.19%	0.19%	0.39%	0.39%	0.39%	0.39%	0.39%	0.79%	0.79%	0.39%	0.39%	0.39%	0.59%		0.0027	0.0035	0.0034	0.0027	0.0027	0.0034	0.0034	0.0019	0.0019	0.0019	0.0027	0.0019	0.0019
Q329_CO1_BhN	0.18%	0.19%	0.92%	0.35%	0.46%	0.39%	0.92%	0.35%	0.39%	0.00%	0.61%	0.00%	0.19%	0.39%		0.0027	0.0019	0.0015	0.0026	0.0017	0.0046	0.0015	0.0048	0.0021	0.0045	0.0021	0.0015
ten10_CO1_BhN	0.35%	0.39%	0.62%	0.53%	0.62%	0.59%	0.62%	0.53%	0.59%	0.18%	0.31%	0.19%	0.39%	0.59%	0.46%		0.0029	0.0023	0.0032	0.0025	0.0039	0.0027	0.0027	0.0027	0.0023	0.0027	0.0027
JN603174Siphonaria_nigerrima	0.39%	0.39%	0.59%	0.59%	0.59%	0.59%	0.59%	0.59%	0.59%	0.19%	0.19%	0.19%	0.39%	0.59%	0.19%	0.39%		0.0019	0.0035	0.0027	0.0040	0.0028	0.0028	0.0028	0.0019	0.0028	0.0028
nig94_BhN	0.18%	0.19%	0.31%	0.35%	0.31%	0.39%	0.31%	0.35%	0.39%	0.00%	0.00%	0.00%	0.19%	0.39%	0.15%	0.31%	0.19%		0.0022	0.0017	0.0031	0.0015	0.0015	0.0015	0.0000	0.0015	0.0015
ten9_CO1_BhN	0.18%	0.19%	0.31%	0.35%	0.31%	0.39%	0.31%	0.71%	0.79%	0.35%	0.31%	0.39%	0.59%	0.39%	0.46%	0.62%	0.59%	0.31%		0.0031	0.0021	0.0015	0.0015	0.0015	0.0022	0.0015	0.0015
EF418596sp	0.35%	0.39%	0.53%	0.53%	0.53%	0.59%	0.53%	0.18%	0.19%	0.18%	0.18%	0.19%	0.00%	0.59%	0.18%	0.35%	0.39%	0.18%	0.53%		0.0040	0.0024	0.0024	0.0024	0.0017	0.0024	0.0024
ann092_CO1_BhN	0.53%	0.39%	0.61%	0.71%	0.62%	0.59%	0.61%	1.07%	0.99%	0.71%	0.61%	0.59%	0.79%	0.59%	1.23%	0.93%	0.79%	0.62%	0.31%	0.89%		0.0040	0.0056	0.0027	0.0059	0.0027	0.0040
Q331_CO1_BhN	0.00%	0.00%	0.61%	0.18%	0.15%	0.19%	0.61%	0.53%	0.59%	0.18%	0.61%	0.19%	0.39%	0.19%	0.15%	0.46%	0.39%	0.15%	0.15%	0.35%	0.92%		0.0041	0.0000	0.0044	0.0000	0.0000
nig012_CO1_RR	0.00%	0.00%	1.24%	0.18%	0.15%	0.19%	1.24%	0.53%	0.59%	0.18%	1.24%	0.19%	0.39%	0.19%	1.24%	0.46%	0.39%	0.15%	0.15%	0.35%	1.56%	0.92%		0.0000	0.0015	0.0000	0.0041
nig11_RR	0.00%	0.00%	0.15%	0.18%	0.15%	0.19%	0.15%	0.53%	0.59%	0.18%	0.15%	0.19%	0.39%	0.19%	0.31%	0.46%	0.39%	0.15%	0.15%	0.35%	0.46%	0.00%	0.00%		0.0015	0.0000	0.0000
nig093_CO_BhN	0.18%	0.19%	1.40%	0.35%	0.31%	0.39%	1.40%	0.35%	0.39%	0.00%	1.08%	0.00%	0.19%	0.39%	1.08%	0.31%	0.19%	0.00%	0.31%	0.18%	1.72%	1.08%	0.15%	0.15%		0.0015	0.0044
ten087_CO1_RR	0.00%	0.00%	0.15%	0.18%	0.15%	0.19%	0.15%	0.53%	0.59%	0.18%	0.15%	0.19%	0.39%	0.19%	0.31%	0.46%	0.39%	0.15%	0.15%	0.35%	0.46%	0.00%	0.00%	0.00%	0.15%		0.0000
Q330_CO1_BhN	0.00%	0.00%	0.61%	0.18%	0.15%	0.19%	0.61%	0.53%	0.59%	0.18%	0.61%	0.19%	0.39%	0.19%	0.15%	0.46%	0.39%	0.15%	0.15%	0.35%	0.92%	0.00%	0.92%	0.00%	1.08%	0.00%	

Shell characteristics

(A) *Siphonaria concinna* and *S. oculus*
Photographs of the shells of *S. concinna* and *S. oculus* specimens employed in my genetic analysis, plus representative voucher shells from the Chambers and McQuaid (1994) revision, are shown in Figures 2.5 to 2.8. For *S. concinna* (Fig. 2.5), the large, "white apical area within the adductor muscle impression" (Allanson 1958, p174) is distinctive, though smaller in young animals. Chambers and McQuaid (1994, p269) also note the "distinct white oval area dorsal to the adductor muscle corresponding to eroded dorsal apex" and that blue flecks are visible on juveniles (Fig. 2.6). On the undersurface the black marginal area is crossed by white radiating stripes corresponding to the costae, and are much narrower than the intervening black spaces between them. The costae project from the shell margin, forming clear indentations between them. Juveniles (Fig. 2.6) are markedly asymmetrical, with a small number of long costae on the right, and a larger number of shorter costae on the left. In adults, the shell becomes almost symmetrical, with a central apex.

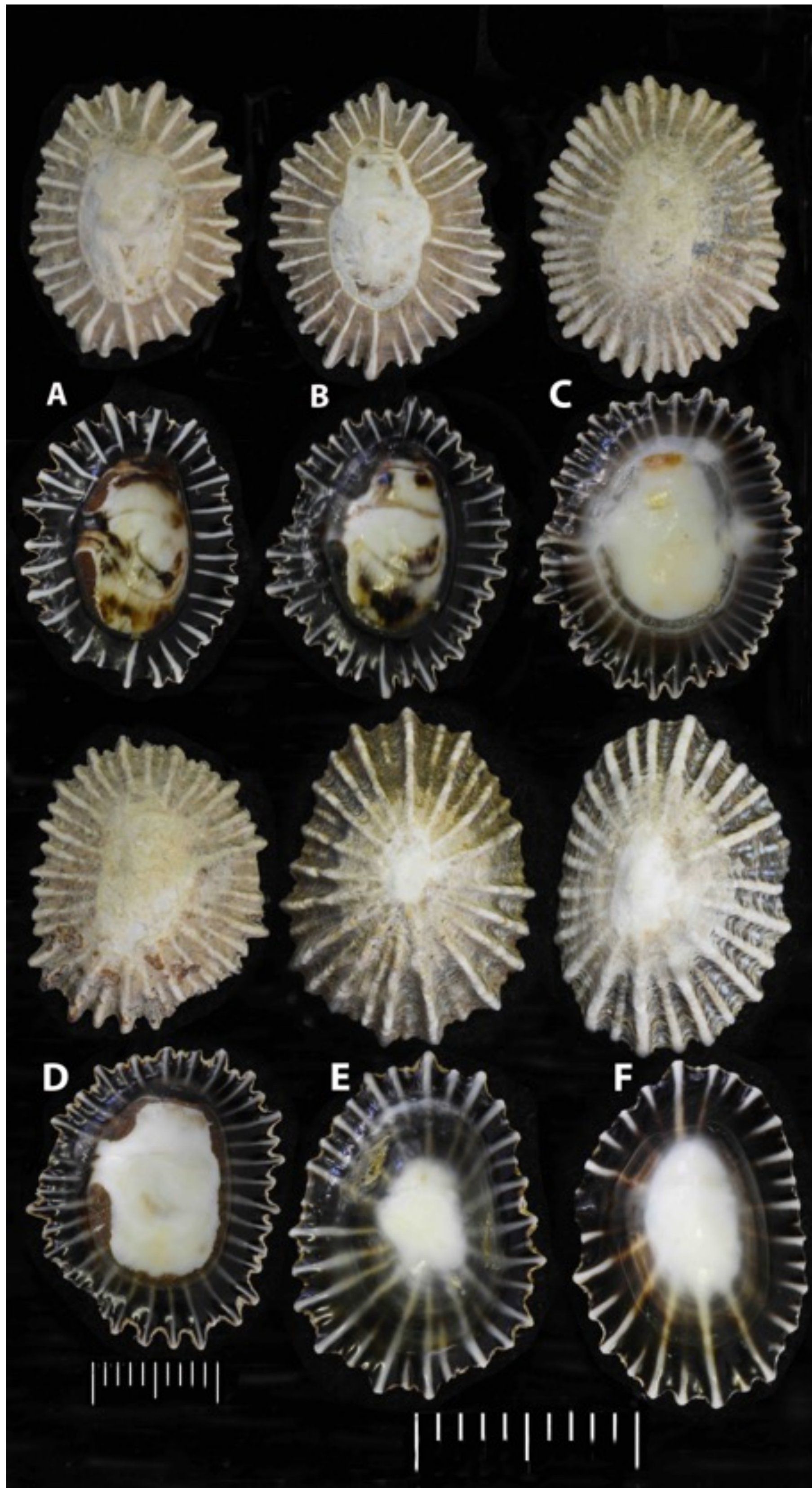

Figure 2.5 Shells of *S. concinna* employed in my genetic analyses, plus representative voucher specimens from Chambers & McQuaid (1994). Upper shell in each pair: dorsal; lower shell: ventral. Specimen numbers as follows: A = 033; B = 032; C = 029; D = 028; E & F = Voucher specimens V50 and V52 housed in KZN Museum. Scale bar = 10 mm. As in all shell plates, dorsal and ventral views are shown.

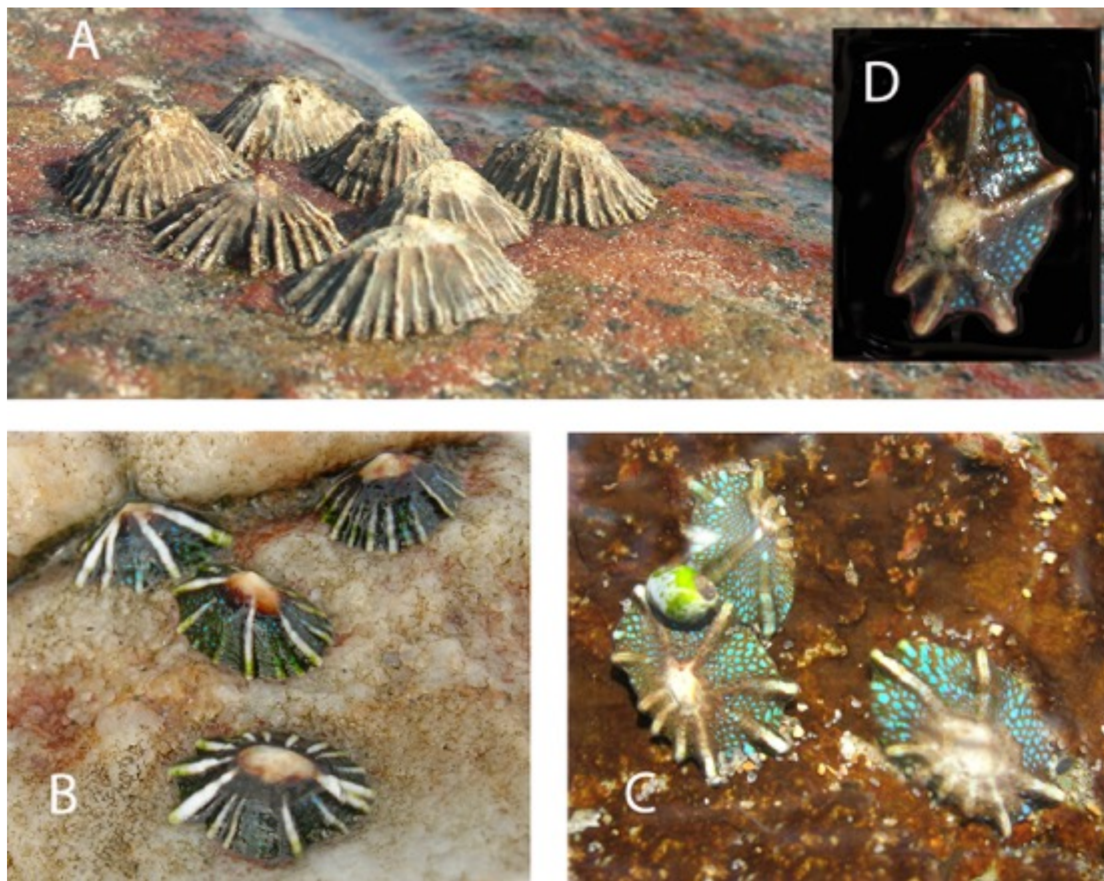

Figure 2.6 In situ photographs of *Siphonaria concinna*. (A): Group of adults; (B): Subadults; (C-D): Juveniles showing iridescent blue-green spots between costae and markedly asymmetric shells. (Images A, B, C courtesy M. Pfaff.)

For *S. oculus*, there is typically a narrow white apical band (B, C, E, G, I in Fig 2.7 and bottom ventral views of smaller individuals in Fig 2.8) on the interior of the shell which becomes more diffuse and less distinctive as the animal gets older and bigger (see J, K in Fig 2.7 and ventral views of the three larger individuals in Fig 2.8). Allanson (1958) refers to this as an internal white transverse bar. As shells erode, this transverse bar is often visible even on the exterior surface of the shell. On the interior of the shell, the white radii at the edge are much shorter than in *S. concinna*, and often roughly the same width as the intervening black background between them. The costae usually do not project from the shell edge to the same degree as in *S. concinna*, so the shells have a more uniformly oval appearance. Numbers of costae do not reliably distinguish the species, ranging from six (in juveniles) to 45 (in adults) in *S. concinna*, and 23 (juveniles) to 60 (adults) in *S. oculus*; but do tend to be more numerous in *S. oculus* when animals of similar size are compared. Juveniles of *S. oculus* are less obviously asymmetrical than in *S. concinna* and the costae more numerous and more equal in size. Flecks of iridescent blue-green are present between costae, but not as numerous or obvious as in *S. concinna* (Fig. 2.6). Adults, however, retain the shell asymmetry, with the apex lying to the left of the mid-line.

I was able to distinguish the two species on the basis of these shell features before undertaking genetic analyses; and in all cases the genetic results confirmed the *a priori* names assigned to specimens.

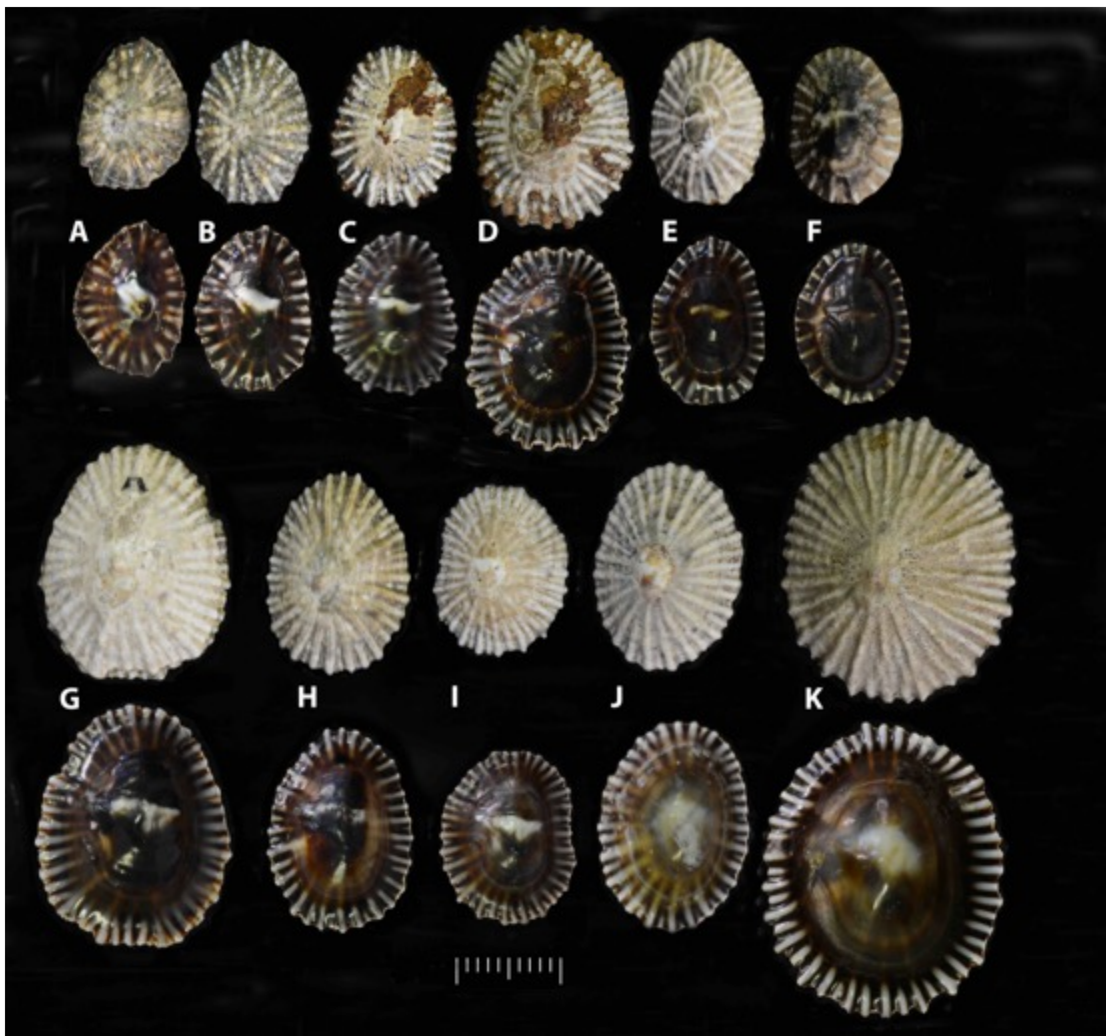

Figure 2.7 Shells of *Siphonaria oculus* employed in genetic analyses. Specimen numbers as follows: A-J, respectively 181, 151, 174, 183, 152, 153, 439, 154, 166, 160. Upper rows: dorsal; lower rows ventral. Scale bar = 10 mm.

Figure 2.8 Voucher specimens V53 of *S. oculus* deposited by Chambers in the KZN Museum. Upper six specimens: dorsal views; lower six: matching ventral views.

(B) The *Siphonaria nigerrima* complex

Fig. 2.9 shows the material that I initially identified as *S. nigerrima*, including specimens of *'S. dayi*', all of which were included in my genetic analyses. For comparison, Figure 2.10 illustrates the paratypes of *S. dayi* from the Iziko Museum and the voucher specimens of that taxon and of *S. nigerrima* deposited by Chambers in the KZN Museum. All of the specimens examined genetically fell in a single clade (Clade C, which fell in the centre of the geographic distribution of the '*S. nigerrima* complex'), despite covering a wide range of shell types spanning from almost uniformly dark blackish-brown, through specimens that were predominantly dark but had a narrow edging of white rays (which would have fallen naturally into the species recognised by Chambers and McQuaid (1994) as *S. nigerrima*) to pale shells with an orange-brown central interior (typical of what was originally described as *S. dayi*). Despite clear differences in shell coloration, I could not distinguish the four putative '*S. dayi*' specimens as being genetically distinct, and it is clear that *S. dayi* must be subsumed under the name *S. nigerrima*.

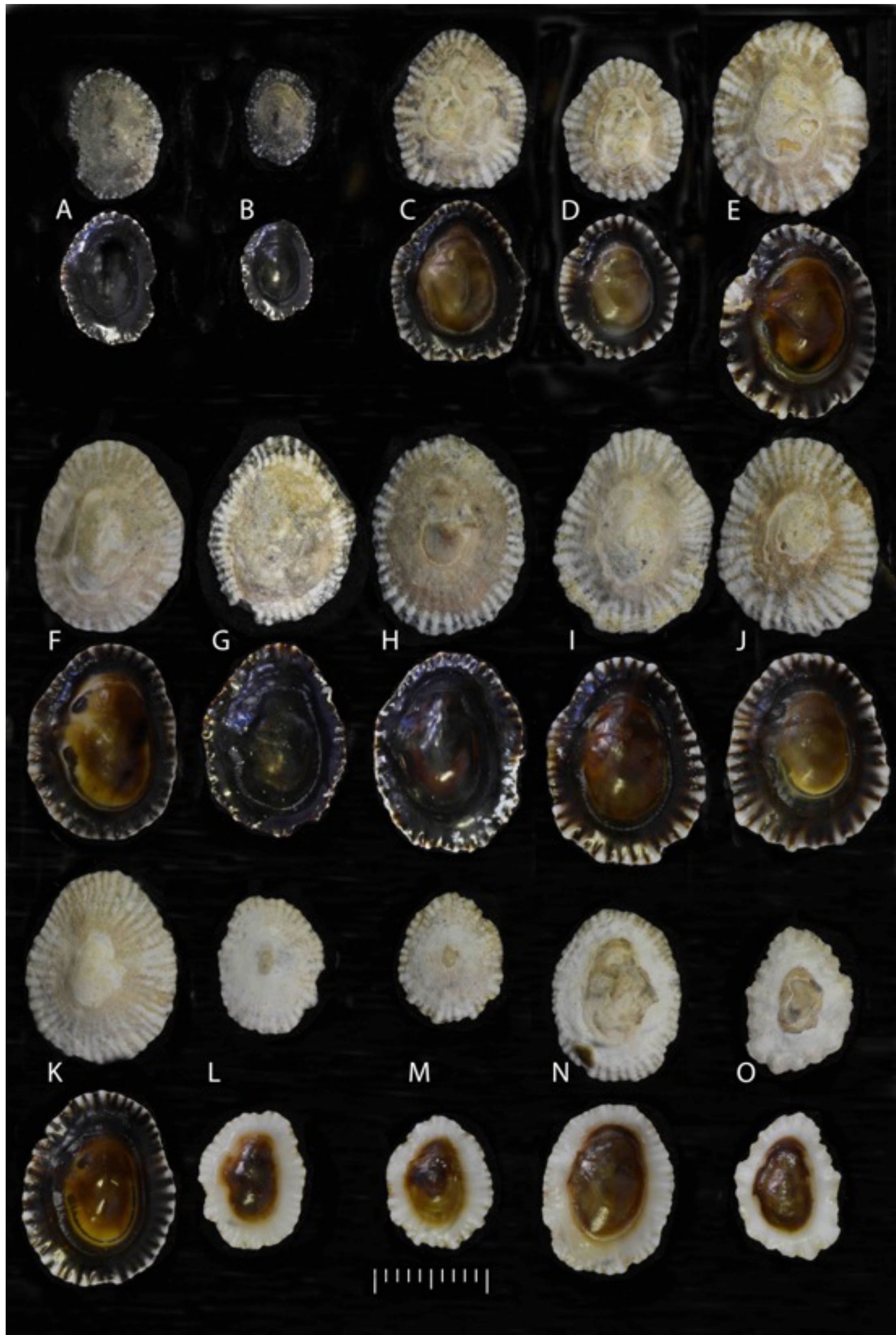

Figure 2.9 Dorsal and ventral views of shells of specimens analysed genetically and falling in Clade C, now assigned to *S. nigerrima*. (A-K): dark varieties previously named *S. nigerrima*; (L-O): pale variety previously named *S. dayi*. Numbers of specimens A-O respectively 011, 012, 89, 90, 91, 92, 013, 94, 329, 330, 331, 009, 010, 87, 88. Scale bar = 10 mm.

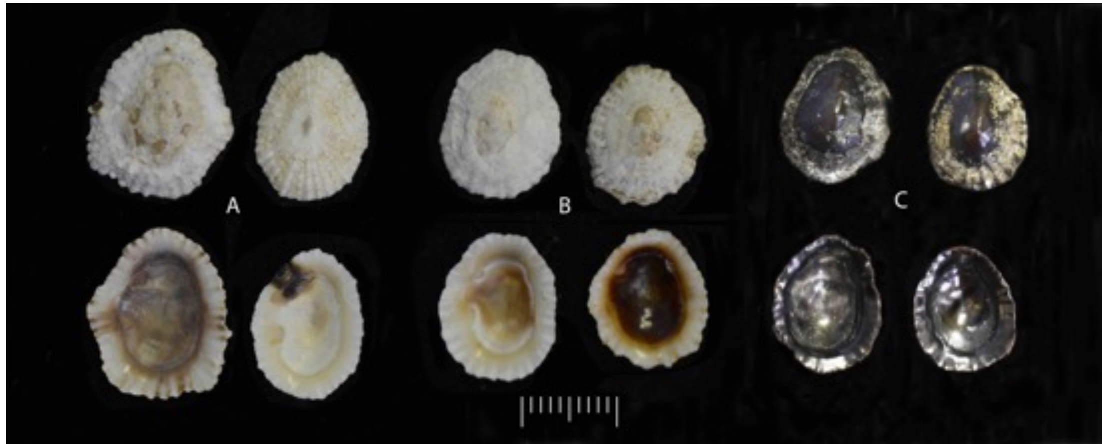

Figure 2.10 (A) Paratypes of *Siphonaria dayi* (ex Iziko Museum), and representative shells from the Chambers & McQuaid revision (1994) of (B): *Siphonaria dayi* sensu stricto and (C): *S. nigerrima* sensu stricto, housed in the KZN Museum. Scale bar = 10mm.

Figure 2.11 illustrates the six specimens from Tofo that emerged as Clade A from my genetic analysis. Smaller specimens were not unlike some of those of *S. nigerrima* in appearance, with a ventral clearly defined central area bordered by the scar of the adductor muscle, and an outer black rim shot with white rays at the edge (compare Figs 2.11A, B with 2.9J, K for example). But larger specimens were different from any of those of *S. nigerrima*, with a diffuse yellow-brown centre with white rays extending from that to the margin. Figure 2.12 shows that this distinctive shell patterning was consistent across other specimens from Tofo that were collected at the same time and locality but not included in the genetic analysis. The muscle attachment in the pallial sinus at the front was indistinguishable in colour from the background colour in nearly all specimens.

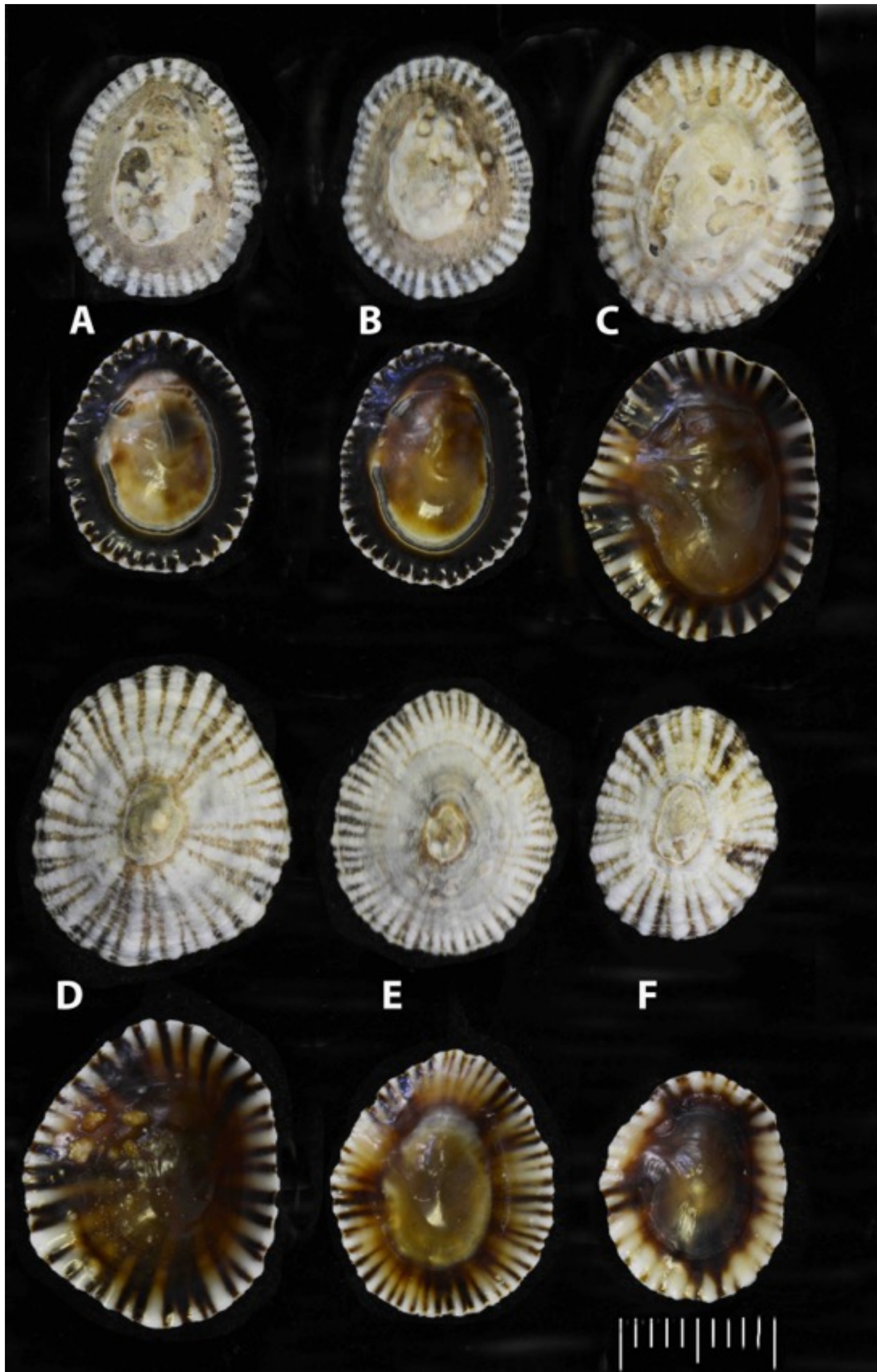

Figure 2.11 Dorsal and ventral views of shells of specimens from Tofo, Mozambique, employed in my genetic analysis and clustering as Clade A. Numbers of specimens A-F: respectively 112, 113, 114, 115, 116, 117. Scale bar = 10 mm.

Figure 2.12 Additional samples of material from Tofo that were not used for genetic analyses, but further illustrate consistency of shell features. Scale bar = 10 mm.

Figure 2.13 shows the two specimens included in the genetic analysis that emerged as Clade B from Chidenguele (Fig. 2.13A, B) and the voucher material Chambers deposited in the KZN Museum and identified by him as *Siphonaria tenuicostulata* (Fig 2.13C, D).

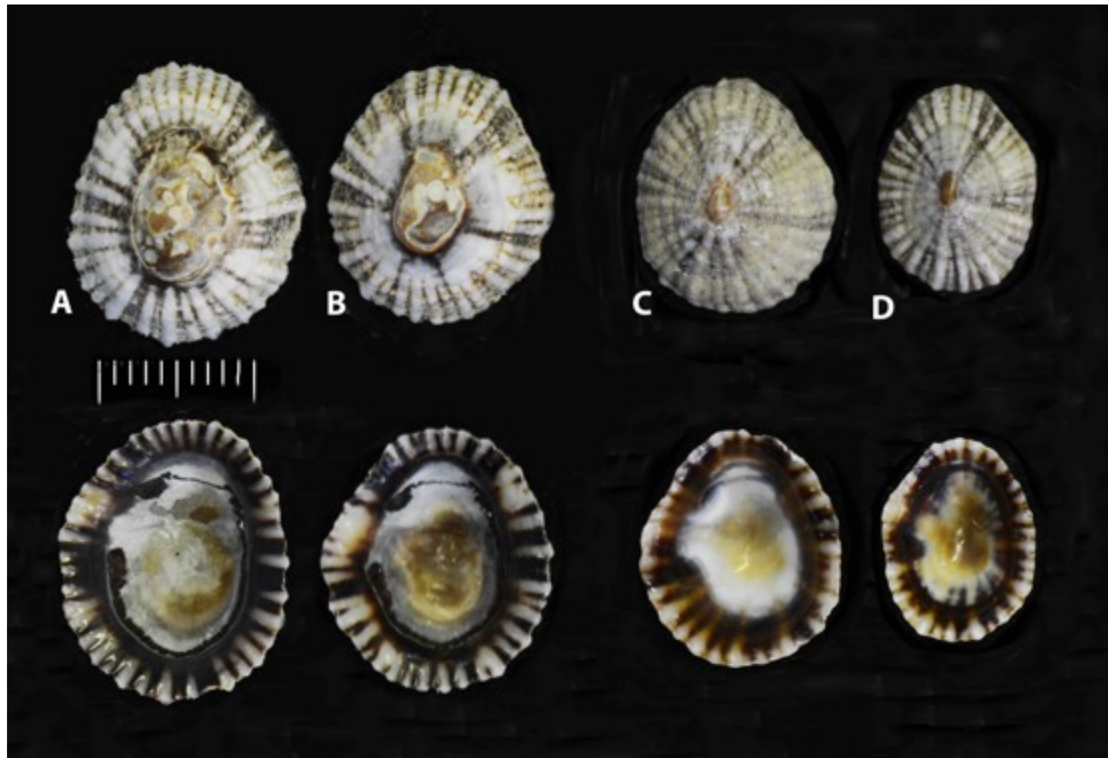

Figure 2.13 (A-B) Shells of genetically analysed specimens from Chidenguele, Mozambique grouping as Clade B and considered to be *S. tenuicostulata*; and (C-D): representative shells of *S. tenuicostulata* from Umhlanga Rocks, Chambers & McQuaid (1994). A-B: Specimen numbers 368 and 369; C-D: Voucher specimens V48 housed in the KZN Museum. Scale bar = 10 mm.

Figure 2.14 adds a series of specimens also collected at Chidenguele but not used for genetic analysis. These shells all bear a close resemblance to the voucher material of *S. tenuicostulata*. The shells can be distinguished by the pale off-white to yellow-green central interior, sharply defined from the surrounding dark black-brown muscle scar, and an encircling black rim that is striped with alternating white and dark rays. The outer edge is predominantly white in smaller individuals but increasingly banded black and white as size increases. The rays are not conspicuous on the exterior of the shell but are still traceable. Two striking features distinguish the shells in this clade: a thin, dark stripe on the lower surface across the front of the pallial sinus where the adductor muscle is attached, and a merging of some of the darker radial rays to create quintuple darker areas around the rim - one each side of the head, one medially on each side just behind the midline, and one posterior-laterally on the right of the undersurface. This fivefold pattern of darker, wider rays is visible even on the upper surface.

Figure 2.14 Additional specimens from Chidenguele not used in genetic analyses, but considered to be the same species and showing the consistency of shell patterning. Scale bar = 10 mm.

The single specimen from Durban (Fig. 2.15A) that was examined genetically and emerged as a divergent genetic lineage (and formed part of Clade D when combined with additional GenBank sequences) had a shell that was indistinguishable from the voucher specimens of what Chambers termed *Siphonaria anneae* (Fig. 2.15B, C). All had a central lower-surface patch of off-white to yellow green surrounded by an oval of black extending to the margin, where it was crossed by short but broad white rays, consistently broader than the intervening black rays. Upper surface often eroded to a central oval pale patch, revealing the white-on-black rays that are more evident on the lower surface. Specimens in this clade (and Chambers' voucher specimens of *S. anneae*) were quite similar to the specimens from Chidenguele, but the quintuple darker rays were absent or much less obvious, the dark line of the adductor muscle across the pallial sinus that characterised the Chidenguele material was absent, and the background ventral colour between the white rays was black rather than dark brown.

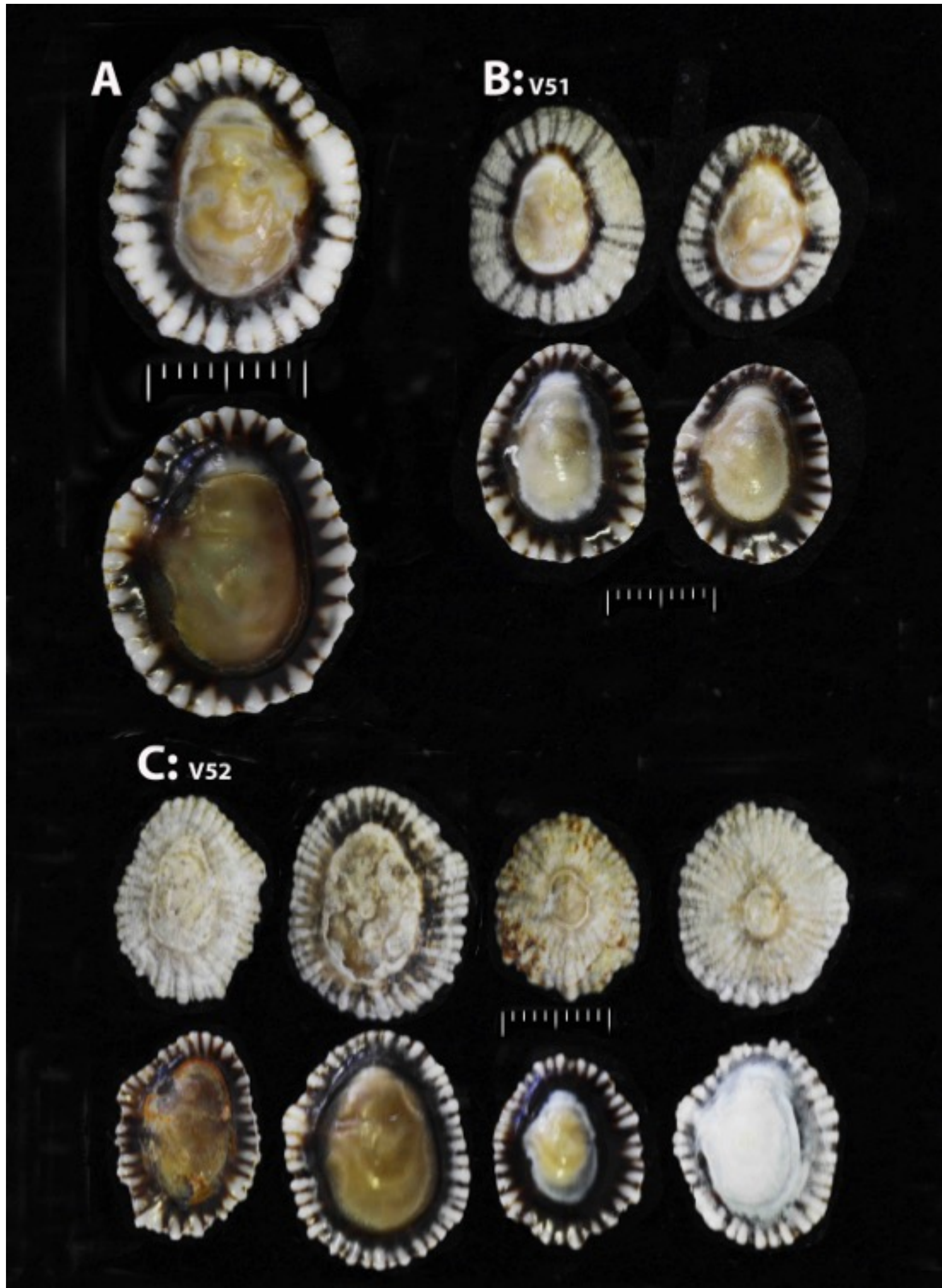

Figure 2.15 (A) Specimen from Durban, South Africa employed in genetic analysis and emerging as the solitary representative of Clade D. (B-C): representative voucher specimens considered to be the same species, named *S. anneae* and deposited in the KZN Museum by Chambers. Specimen numbers: A = 182; B = V51 (Umhlanga Rocks); C = V52 (Umhloti). Scale bars = 10 mm. Note differences in scale between A and B-C.

Finally, the three specimens from Mkambati that clustered in Clade E (Fig. 2.16A-C) and one other collected at the same time and locality but not included in the genetic analysis (Fig. 2.16D), were also distinguishable morphologically. The interior of the lower surface is greenish-pink in small individuals but darkens to almost continuously black, with very short marginal white rays. The costae project moderately from the margin – more so than in members of the other clades - and the lobe accommodating the siphon is more pronounced, at least in larger individuals.

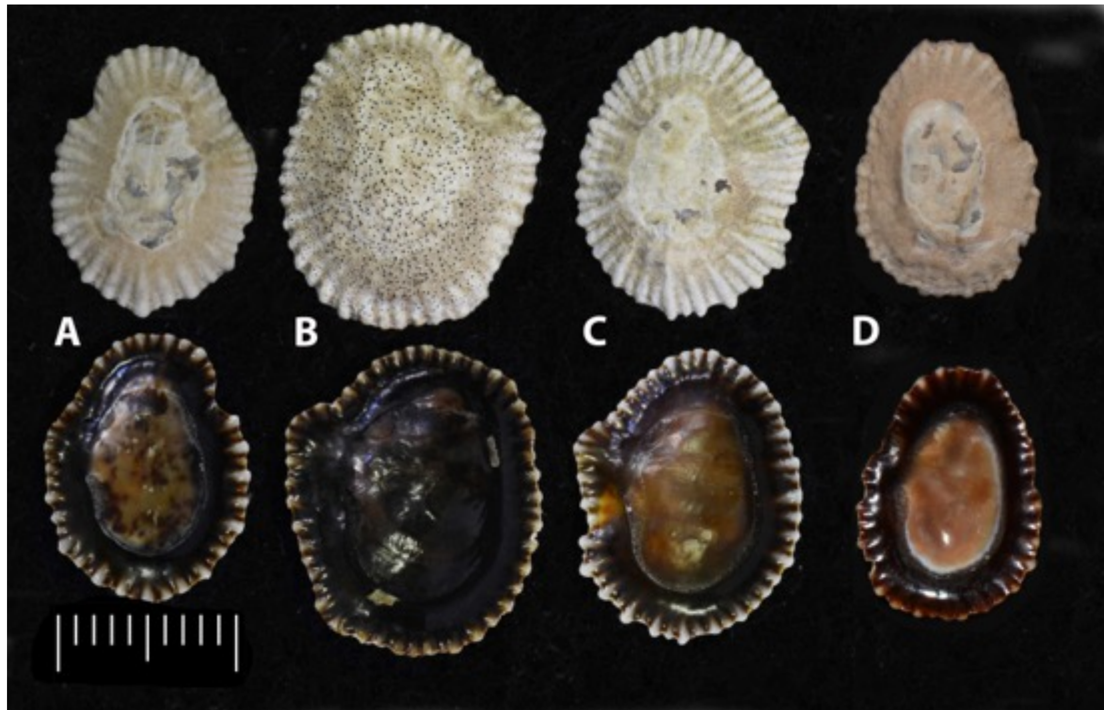

Figure 2.16 (A-C) Specimens from Mkambati, South Africa, used in the genetic analysis and clustering as Clade E. (D) An additional shell from the same site not examined genetically. Specimen numbers for A-C respectively 181, 428, 429. Scale bar = 10 mm.

In relation to the question of whether material classed as *S. nigerrima* should more correctly be termed *S. carbo*, Figure 2.17A-C shows the type specimen of *S. carbo* from the Natural History Museum, London. It does not bear any resemblance to any of the specimens I examined in the *S. nigerrima* complex. Salient differences include the 'stepped' growth rings, the chocolate brown interior colour and the size of the specimen (22 mm shell length, exceeding the maximum of 18 mm I recorded for *S. nigerrima*). Indeed, the type of *S. carbo* most closely resembles *Siphonaria zelandica*, with strong concentric growth rings and similar uniformly brown interiors (Fig. 2.17D-E). However, as the type locality of *S. carbo* is unknown, and it is impossible to assess its genetic composition, no secure connection can be made between these two species.

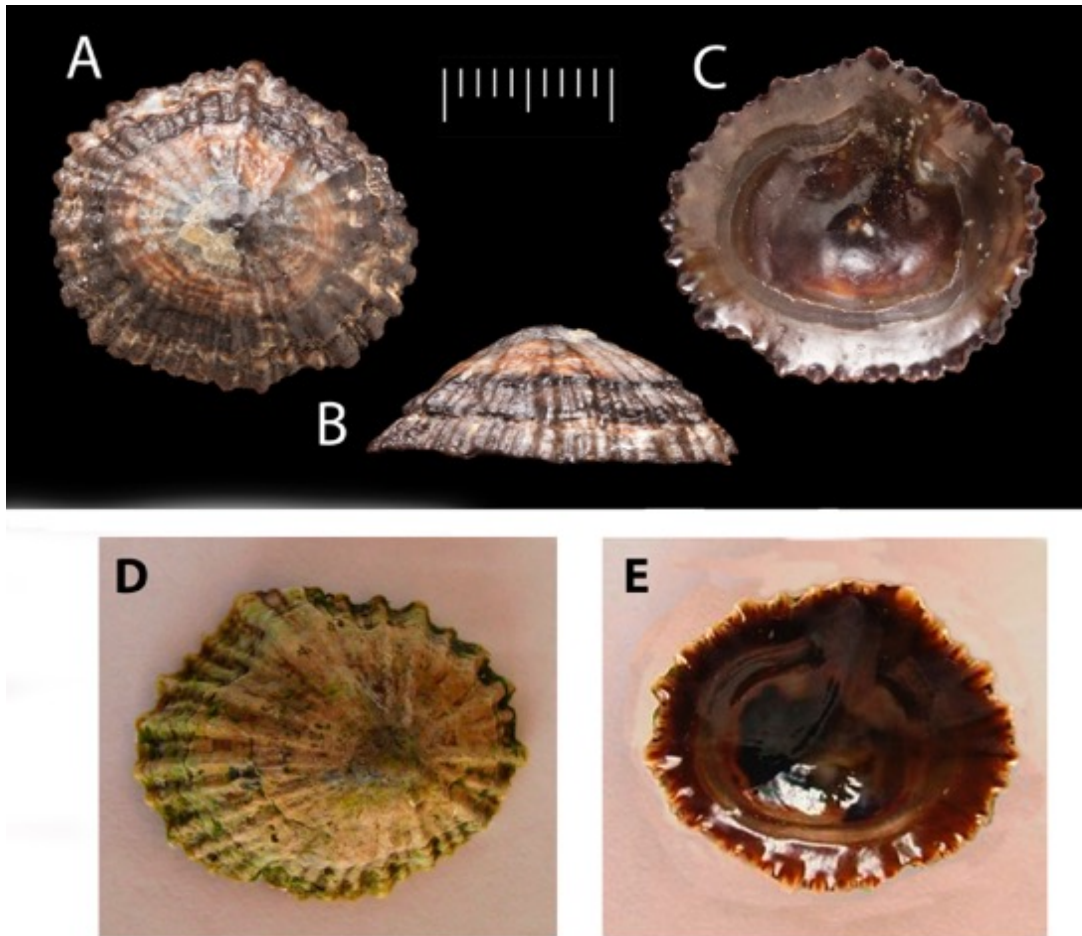

Figure 2.17 (A-C): Dorsal, lateral and ventral views of type specimen of *S. carbo* (photograph courtesy of Jon Ablett and the staff of the Natural History Museum, London)(Scale bar = 10 mm) and (D-E): of *S. zelandica* (photographs by Graham Bould: *Siphonaria zelandica* from Whatipu, New Zealand https://en.wikipedia.org/wiki/Siphonaria_zelandica: Public Domain, https://commons.wikimedia.org/w/index.php?curid=4755217. Sited identifying source: Powell A. W. B., *New Zealand Mollusca*, William Collins Publishers Ltd, Auckland, New Zealand 1979 ISBN 0-00-216906-1)

2.4 DISCUSSION

Classical molluscan taxonomy is based largely on shell morphology (Puillandre *et al.* 2012). One of the major ongoing problems researchers face is that, due to the high degree of phenotypic plasticity (Bickford *et al.* 2007), shell morphology can be misleading when identifying members of a group (Fedosov *et al.* 2011). This is particularly true of members of the Siphonariidae.

Some research suggests that morphology is subject to environmental conditions to the extent that morphometric analyses of shell shape cannot distinguish species. For example, Mauro *et al.* (2003) were not able to distinguish three sympatric species of Mediterranean limpets. The rough periwinkle *Littorina saxatilis* is another example of

an extremely variable species exhibiting considerable differences in shell size, colour, and thickness among ecotypes inhabiting different shore microhabitats. As such, it is deemed a possible example of incipient speciation (Johannesson *et al.* 2010) or incomplete ecological speciation (Tirado *et al.* 2016).

These and many other issues have led to debate on what constitutes a species and the status of subspecies and varieties in the Mollusca and make the group a perfect candidate for genetic resolution. *Siphonaria* is no exception and shell variability remains a significant problem. In addition to intrinsic variability among individuals, and between individuals at different shore levels, adult and juvenile shells are often very different in shape and colour as can be seen in specimens of *S. concinna* (Figure 2.6). In addition, shell erosion caused by waves, sand and drilling of the shell by endolithic commensals such as cyanobacteria and lichens can reduce distinguishing features (Kaehler & McQuaid 1999, Zardi *et al.* 2009) (Figure 2.16B).

These issues make the genus ripe for molecular investigation.

Outcomes emerging from my analyses

Four different issues have been addressed in or emerged from this chapter. Firstly, the confirmation of the species status of two existing species, *S. concinna* and *S. oculus*; secondly, the confirmation of the merging of two formally accepted species (*S. nigerrima* and *S. dayi*); thirdly, the emergence of undescribed clades/species identified by genetic means and, fourthly, clarification of specific names assigned in publications and online databases.

With regard to the first issue: the names of *S. concinna* and *S. oculus* remain accepted as separate species and are not changed. The second to fourth issues require name assignments to be addressed (a) for the species group made up of *S. nigerrima* and *S. dayi*, currently formalised as *S. carbo,* (b) what names should be attached to putative new species, and (c) corrections of names in databases.

Clarification of S. concinna and S. oculus as separate species

Siphonaria concinna and *S. oculus* were resolved as two species, identifiable by both DNA sequence and shell differences, specifically the internal colouration. Initially I had no difficulty differentiating between the two morphologically, and my identification based on shell morphology were subsequently confirmed by the genetic analysis.

This is in agreement with Allanson (1958) who identified them as two distinct species on the basis of the distal genitalia, and Chambers & McQuaid (1994) who made use of protein and DNA fingerprint methodology. Hubendick (1946) did not consider there to be sufficient differences between the two to consider them distinct species.

Teske *et al.* (2011) identified two distinct evolutionary lineages in *S. concinna,* one of which was confined to south of the Cape St Lucia phylogenetic break. However, after incorporation of their GenBank data into my COI dataset it appears that certain of their sequences were incorrectly assigned as *S. concinna* and were in fact *S. oculus* (Appendix

2.2). No samples labelled or identified as *S. oculus* were included in their work. As no shells associated with their samples used for genetic analysis were available for examination, this conclusion is based on the clustering of their sequences with specimens identified by myself as *S. oculus*. This suggests that their identification of two evolutionary lineages in what they considered a single species was in fact due to differences between two already described species. It was possible to identify from their work exactly where each sample fell geographically and thus determine that only sequences from individuals sampled from localities south of Cape St Lucia occurred together with animals that I identified as *S. concinna,* while individuals from both north and south of Cape St Lucia grouped together with animals identified as *S. oculus* (Fig 2.3B), thus agreeing with the accepted distribution for these two species (Fig 2.1, Branch *et al.* 2016). The genetic division is, nevertheless, interesting, as it accords with a biographic break-point at that locality identified by Sink *et al.* (2005) and Porter *et al.* (2017)

Siphonaria dayi, S. nigerrima, S. anneae and S. tenuicostulata

Based on DNA fingerprinting, Chambers *et al.* (1996) argued that the close genetic similarities between *S. nigerrima*, *S. anneae* and *S. tenuicostulata* indicate a recent common ancestry and that the sympatric sister taxa *S. anneae* and *S. tenuicostulata* are probably relatively young species possibly evolved from the sympatric *S. nigerrima*. *Siphonaria dayi* was found to be more distantly related and parapatric to all three and possibly the clade ancestor (Chambers *et al.* 1998, p62). They did note that variation within the genus and between subgenera may be very small. In contrast, Teske *et al.* (2007) provided evidence that *S. anneae, S. tenuicostulata, S. dayi* and *S. nigerrima* were a single species. They also found that specimens of the four species collected on either side of the Cape St Lucia biogeographic disjunction formed distinct monophyletic clusters. Later work on the conflated *S. nigerrima* (comprising the lumped-together *S. anneae, S. tenuicostulata, S. nigerrima* and *S. dayi*) identified two distinct lineages among the haplotypes (Teske *et al.* 2011).

Based on a mitochondrial locus of 1130 base pairs for the 16S+COI dataset (and confirmed with the expanded COI tree) I found evidence for at least four, and probably five, clades in a polytomous tree. Utilising Chamber's voucher specimens in the KwaZulu-Natal Museum as reference material, I found that the specimen from Durban (identifiable as *S. anneae* from the voucher material: Fig. 2.15) and specimens from Chidenguele, Mozambique (corresponding to voucher material of *S. tenuicostulata*: Figs 2.13 and 2.14), resolved into two of those clades (Fig. 2.3B; Clades D and B respectively). This leads me to suggest that both *S. anneae* and *S. tenuicostulata* are valid species, and are identifiable from shell characteristics alone. Material united in Clade D extended from Durban to Mission Rocks. Clade B, which I consider to be *S. tenuicostulata*, is unlikely to be limited to the Chidenguele location from where my material came. The type locality of the species is Umhlali 40 km north of Durban, and Chambers *et al.* (1998) identified this species as being present at Umhlanga, 25 km SE of

that, thus overlapping with the distributions of Clade C (*S. nigerrima*) and Clade D (which I provisionally consider to be *S. anneae*). Richmond's (2011) record of the species in the Western Indian Ocean to the Gulf of Arabia radically extends its potential distribution.

A distinct and large clade (Clade C) was made up of *S. nigerrima* together with what would previously have been identified as *S. dayi* according to the voucher material, with no difference evident between them bar shell differences (Figs 2.3B, 2.9). This clade also has the highest genetic within-group distances (0.38%) and the widest range among individuals within the same clade (0 – 1.72%) (Table 2.3a, c). The highest genetic difference in the entire dataset was between two individuals from this clade both collected from the same site (Bhanga Neck), to which I initially assigned different names. I would suggest that this result indicates a possible anti-cryptic pattern, which would require a finer method of molecular differentiation to resolve adequately. Until such time, *S. nigerrima* and *S. dayi* appear to be one and the same species, however unlikely that appears from their shell morphology and from the high within-group and within-species percentages for the clade compared to the other clades, and *S. dayi* should be subsumed under *S. nigerrima*. Distribution of this clade ran from Jesser Point to Bhanga Nek, thus lying north of Clade D.

In addition, two novel clades, one from individuals sourced from Mkambati, eastern South Africa (Fig. 2.3B, Clade E; Fig. 2.16) and the other from Tofo, Mozambique (Fig. 2.3B, Clade A; Figs 2.11 and 2.12) were clearly resolved. I originally identified them as 'problematic' in terms of assigning them to existing described species, hence their being labelled 'Q' prior to sequencing.

There are several reasons why some of the conclusions of Teske *et al.* (2007) may differ from mine. Firstly, their study included a nuclear locus, ATPSβ instead of the mitochondrial 16S locus. Secondly, the specimens were sampled only from the geographic range corresponding to *S. nigerrima* and *S. anneae* in my study, and they sequenced 6 unique COI haplotypes. Five of these COI haplotypes are identified in my Appendix 2.2 as *S. nigerrima* for both their original species name and the correct name. However, one of these COI haplotypes (EF418591) clusters in Clade D in Fig. 2.3, and therefore the correct identification for that individual is *S. anneae*. Teske et al. (2007) originally identified all specimens from Umhlanga Rocks and two individuals from Blythesdale Beach as *S. anneae* (and the illustrated shells appear to be consistent with that identification), so haplotype EF418591 most likely corresponds to those individuals. Other shells from Blythesdale Beach in that study differed in morphology from *S. anneae* and were identified either as *S. nigerrima* or *S. tenuicostulata* based on both COI and the nuclear locus ATPSβ from those individuals. Incidentally, a further three individuals, identified as *S. nigerrima* in Teske *et al*. (2011): JN603175 (Mission Rocks), JN603177 (Zinkwazi) and JN603178 (Blythedale), also cluster in Clade D (Fig. 2.3, App. 2.2). In summary, my study differs from Teske *et al.* (2007) in regarding *S. anneae* and *S. tenuicostulata* as distinct species that can be distinguished genetically from *S. nigerrima,* but my data on *S. dayi* support the view of Teske *et al*. (2007) that that morphotype is genetically indistinguishable from *S. nigerrima*).

Although Teske *et al.* (2007) make the point that the combined genetic diversity of the four species making up the *S. nigerrima* complex fall within the range of a single species and is lower than that in other southern African *Siphonaria*, I found that the genus contains recognised species with both higher (*S. serrata, S. capensis* and *S. compressa*) and lower (*S. concinna* and *S. oculus*) nucleotide diversity (Table 2.2).

It thus appears that *S. dayi* (as originally described) is not distinguishable genetically with my locus even though there is a clear difference in phenotypic characteristics between it and other examples of *S. nigerrima* and some indication from my genetic distance measurements that there is more to be uncovered. In a similar scenario for birds, the reasons given for this is that morphological variation can exist without being reflected in the mitochondrial DNA structure because (a) these morphological traits are polygenic, (b) they are under selection and (c) evolve faster than do neutral DNA markers (Zink 2004, Zink & Barrowclough 2008). In another problematic group, the haplochromine cichlid species-flock of Lake Victoria (a group that is morphologically and behaviourally differentiated from a few closely related founding lineages), genetic diversity was found to be high at the 11 loci of the 6 species studied, but there was no evidence of genetic differentiation between the species (Samonte *et al.* 2007). This undifferentiated, shared genetic variation was thought to be due to a recent origin of the radiation and ongoing hybridisation, which would maintain the shared polymorphism via incomplete lineage sorting. However, later work using much more Next Generation Sequence (NGS) data (in the form of supermatrices of genome-wide sequence data) provided evidence of reciprocal monophyly with strong support for the 16 morphologically defined sympatric species studied in a single area (Wagner *et al.* 2013).

The precedent has thus been set for rapidly evolving polygenic morphological traits under selection that are only discernable with more sensitive methods. Given the high number of site-specific haplotypes identified (Figure 2.4), the additional new clades found at Mkambati and Tofo, the anti-cryptic nature of *S. dayi* (Figure 2.3B), and the distinct monophyletic clusters and the two haplotype lineages identified by Teske *et al.* (2007, 2011), I suggest what is needed to fully resolve the east coast species is an NGS project entailing much greater sampling effort and extending from the south east of South Africa through to northern Mozambique and possibly even further north up to the Arabian Sea.

Additional reasons for more work here and even further north are (1) Richmond's (2011) observation that *S. tenuicostulata* and three other similar species are present in the Western Indo Pacific, extending as far as the Gulf of Aden, and (2) Reid's contention that there are more un-named species on the east coast of Africa. It is likely that only a methodology more sensitive than sequencing single genes will be successful in clarifying this situation – as has been the case for the cichlid fish of Lake Victoria.

Nevertheless, my data are strong evidence that *S. nigerrima* and *S. dayi* are conspecific.

Clarification of the specific name for S. nigerrima and S. dayi

First, I consider *Siphonaria carbo*, the name that has been adopted in Catalogue of Life and MolluscaBase for the four currently merged species *S. nigerrima*, *S. tenuicostulata*, *S. anneae* and *S. dayi*. In Hanley's (1858) description of *S. carbo* he states: "The only individual known to me might pass externally for *S. brunnea*, and internally for a dwarf *characteristica*. From the former it is readily distinguished by the uniform and intense darkness of its somewhat bronzed interior, from the latter by its very numerous and close set ribs which are apparently equal in breadth and more or less blunt" (Hanley 1858, p24). No locality is given: just that it comes from the Cuming collection ("Mus. Cuming"). No plates or figures are associated with this description. The details of measurements in the description are "11 x 9.5 lin." with no further explanation provided. The unit 'lin.' is problematic as it has various interpretations, but is usually taken to be = 2.25 mm in botanical circles or 2.11 mm in British usage, making the specimen 22.5 or 23.2 mm in length. From the scale provided for the type specimen is in the Natural History Museum, London (Figure 2.17A-C), its length is 22.8 mm. The size of the type specimen is sufficiently greater than any of the material I considered to be *S. nigerrima* (mean 13.4 mm, maximum 17 mm) to make it unlikely that the two are the same species, and in appearance, it does not correspond well with any of the southern African specimens in the *S. nigerrima* complex. However, as *Siphonaria* shells are notoriously variable, and in the absence of any material that would permit genetic analysis of the type specimen, it is impossible to be certain about this. On the basis of the shell alone, the type of *S. carbo* bears a strong resemblance to a shell of *S. zelandica* from Whatipu, New Zealand (Figure 2.17C,D).

With regard to the two species mentioned in the original description of *S. carbo,* the first, *Siphonaria brunnea* (now deemed a synonym of *S. alternata* by some: see gastropods.com) was described by Hanley (1858) just above *S. carbo* on the same page of the original publication. It is smaller than *S. carbo* (10.5 x 9 lin.), and its locality given as "In insula Bermuda. Mus. Cuming" (Hanley 1858, p24). The second of these species, *Siphonaria characteristica* from Panama, was described from a specimen 22.5 x. 18.75 x 12.5 mm (Reeve 1842a) "approaches very closely to the *Siphonaria gigas* of Sowerby" (Reeve 1842b, p 50). The figure drawn (internal view only) in Reeve's (1842a) Plate 138, Figure 3 looks similar to the Natural History Museum photo of *S. carbo* in terms of colour but if the drawing is to scale then the shell is over 50 mm in length, which would conform with it being *S. gigas*, as that is the only species in the genus *Siphonaria* to reach that size. The appearance of the shell (as illustrated in Reeve 1856, Plate II, Figs 8a, b) is, however, very different from a typical *S. gigas*, with multiple narrow ribs rather than a few major ribs. Clearly it was the colour Hanley was referring to in concluding that *S. carbo* resembled *S. brunnea*. I think Hanley was therefore most likely describing a dark variety of *S. alternata.*

Under Hubendick's (1946) recording of *S. alternata* he refers to three variants from Bermuda distinguished by Davis (1904), one of which is *S. alternata* var. *opalescens* (now a synonym of *S. alternata*): "a relatively small shell, length 16 breadth 13 mm,

blackish, darkly opalescent, rare" (p45). The original description states: "Shell small (size 16 x 13 mm), blackish, opaque, *opalescent,* rare, Hungry Bay, south shore, (Fig. 15)" (Davis 1904, p127). Unfortunately, the photograph of the shell on Plate 4, Figure 15 of the Davis original shows a rather pale interior and the described darkness is not evident. However, from this I gather that dark forms of *S. alternata* do occur. *Siphonaria opalescens* is currently classified as *taxon inquirendum* in WoRMS and MolluscaBase.

These are the only facts I have been able to gather regarding *S. carbo*. No type locality is given in the original description and there is no obvious connection with South Africa. Morphologically, its shell does not resemble specimens in the *S. nigerrima* complex. I believe there is a plausible connection with *Siphonaria alternata* but can find no support for applying the name *S. carbo* to any southern African material.

Chambers & McQuaid (1994), after examining the Natural History Museum holotype of *S. carbo* (Figure 2.17), concluded that *S. carbo* does not occur in South Africa, but suggested it may occur in the Caribbean, because Hanley's description of *S. carbo* reads "the only individual known to me might pass externally for *S. brunnea*…" (1858, p24)" which is described as coming from the Caribbean – just before the description of *S. carbo* on the same page. Reid's unpublished commentary (dated 1995, p20) agrees with Chambers & McQuaid (1994) that *S. nigerrima* is not *S. carbo*. He writes that *S. carbo* has a "robust, thick, shell and is perhaps best related to e.g., *S. (Siphonaria) alternata* Say, 1826, from the Caribbean; this is confirmed by Chambers & McQuaid, (1994)". He writes further: "Smith's 1903 comment re the possibility of *S. nigerrima* being a juvenile form of *S. carbo* has led to numerous mislabelling errors in collections, and to incorrect distribution and description records" (Reid, 1995, p23). As an aside, if Smith considered *S. nigerrima* as a juvenile of *S. carbo*, that would also explain why the type of *S. carbo* is so much larger than any of the material I examined in the *S. nigerrima* complex.

I agree with both Reid (1995) and Chambers & McQuaid (1994) that the name *S. carbo* is not applicable to southern African material, and turn now to addressing the four species names that have been synonymised by Teske *et al.* (2007) as *S. nigerrima*.

Smith (1903, p356) describes *S. nigerrima* (10 x 8 x 4-5mm) from "Umhlali, Natal (Burnup)" and his figures 4 and 5 in Plate 15 look like the darker forms of *S. nigerrima*. He writes "Remarkable for its coal black colour within and without … Perhaps the young condition of *S. carbo*, Hanley, but the costae appear rather finer", thus implying that the *S. nigerrima* examined were smaller than the type of *S. carbo.*

Smith's description of *S. tenuicostulata* (18 x 14 x 6.5mm) and his Plate 15 Figures 14 and 15, also from Umhlali, Natal (Burnup) look like what I would have identified as *S. tenuicostulata*.

In Tomlin's original description of *S. anneae* he quotes from a note received from Professor and Mrs Stephenson: "After comparing the Isipingo material with hundreds of *Siphonaria* shells collected from all round the South African coast (and including a wide range of varieties of *S. oculus*), we concluded that *S. anneae* (15 x 13 x 5.5mm) must be a species distinct from any of the other common South African forms" (Tomlin, 1944, p92). Stephenson's note to Tomlin further describes it as "consistently smaller than the other species with which it associates" (Tomlin, 1944, p93).

Hubendick (1946) never mentioned *S. anneae*, possibly because he was unaware of Tomlin's 1944 description as his work was published at about the same time. Some of Hubendick's other descriptions of the species dealt with from the east coast of South Africa are inconsistent. He refers to Smith's (1903) plate 15 figures 4 and 5 of *S. nigerrima* as the original source for his description of *S. carbo* while at the same time making *S. nigerrima* a synonym of *S. carbo* (Hubendick 1946, p35). It appears he subscribes to the name *S. carbo* but points to Smith's description and figures of *S. nigerrima* for support, possibly because there is no figure associated with Hanley's (1858) original description of *S. carbo*. In his key, for *S. carbo*, Hubendick (1946, p13) has "Shell dark brown or entirely black externally and internally". He is clearly describing what I would call *S. nigerrima*, and his own photograph (Plate 6, figures 16 and 17) appears to be of *S. nigerrima*. He describes the shell margin as "distinctly scalloped" – not a characteristic of *S. nigerrima*.

In Hubendick's recording of *S. tenuicostulata* just below that of *S. carbo*, he also refers to Smith's original 1903 description for *S. tenuicostulata* (Plate 15, figures 14 and 15) and he describes the shell as "medium large" – the same words he uses to describe *S. concinna* and *S. capensis*, which are distinctly larger species. Again, this is not a feature of this smaller species. For both *S. carbo* and *S. tenuicostulata* he states: "Specimens examined: Umhlali, Natal, about 10 sps. in alc. borrowed from Dr H. Watson, Cambridge." (Hubendick 1946, p35). Hubendick's own photograph of *S. tenuicostulata* (Plate 6, figures 16 and 17) does appear to be that of *S. tenuicostulata* as I understand it. To conclude, Hubendick's photographs and descriptions (with some exceptions – notably his size estimations) appear to be of what I would call *S. nigerrima* and *S. tenuicostulata.* However, I believe he focused on the comment by Smith (1903, p356) "Perhaps the young condition of *S. carbo*" and the description by Hanley (1858, p24) of *S. carbo* as "uniform and intense darkness of its somewhat bronzed interior" when describing *S. nigerrima.* This may have led him to choose the name *S. carbo* over *S. nigerrima.*

Allanson (1958) believed *S. tenuicostulata* to be a synonym of *S. anneae*. He notes that Hubendick's "description and figures agree with the material listed as G. 10 A in the collection of the Department of Zoology, University of Cape Town from which Tomlin's types were taken" (p167), the only difference being in rib number. (I have not been able to locate this material.) He reports that the distal genitalia are "exactly similar" in the two species (p167).

Allanson examined neither the type material nor the original description for *S. carbo* but felt that Hubendick's description and figure "correspond to some degree with the material studied from Inhaca Island" (p169) and so relied on correspondence with H. A. Rehder, Curator Division of Mollusks from the Smithsonian Institute who agreed with him that the material Allanson studied from Inhaca Island "is as far as he could determine a young stage of *S. carbo*" (Allanson 1958, p169). Again, the inference is that Allanson's material was smaller than the type of *S. carbo*.

Allanson (1958) does not reference Smith (1903) in which both *S. nigerrima* and *S. tenuicostulata* are first described and, even though Hubendick discussed *S. nigerrima*

(as a synonym of *S. carbo*), Allanson overlooked it. Allanson thus, I assume, never saw the original description for *S. nigerrima* and by his own admission did not have Hanley's original description and did not see the type material for *S. carbo*. I therefore believe he did not have sufficient evidence to make an informed decision about *S. carbo*. Had Allanson known of the *S. nigerrima* description or seen the type for *S. carbo* I believe he would not have used the name *S. carbo*.

While the names *S. anneae*, *S. tenuicostulata*, *S. nigerrima* and *S. dayi* have currently been accepted as synonyms of *S. carbo*, I argue that *S. nigerrima* should be the name for the merged *S. nigerrima* and *S. dayi* rather than *S. carbo* for the reasons outlined above.

New species?

Shells that I identified as failing to fit known descriptions (queries designated 'Q') did resolve themselves into two separate clades that were distinct when I examined them genetically. Teske *et al.* (2007) sampled at four sites (Inhaca Island, Sodwana, Blythedale and Umhlanga) when collecting for the research in which they merged the four species. The two new taxa recorded in my genetic analysis came from Mozambique north of where they sampled (Tofo) and Mkambati, south of their sampling area. This leads me once again to suggest that the whole area of the east coast of southern Africa from Port Alfred but especially north of the Mozambique border and extending up to Tanzania and Kenya needs further investigation and a thorough sampling by increasing the numbers of individuals sequenced and numbers of sites sampled. I suspect that will result in the discovery of many more clades and many more haplotypes.

Identification of appropriate specific names and a formal description of these two clades will have to await such further sampling and research.

Clarification of invalid names currently applied to South African species

Finally, I comment names allocated to southern African species that I do not consider to be valid, but which are still listed as being here in numerous websites and databases such as MolluscaBase, WoRMS and Catalogue of Life.

(A) *Siphonaria adjacens* Turton 1932

Turton (1932, p9) writes "it is probably a new species, but as I only found a single specimen I hardly like to name it as such" and refers to it as a new variety, "*Siphonaria concinna adjacens* (21 x 17 x 5.5mm)", and not as a new species. From Plate 2, no. 75, in Turton's book, I consider that it falls within the range of varieties of *S. concinna* and does not warrant a subspecific or varietal name. MolluscaBase registers it at a specific level as a *taxon inquirendum*, and does not accept the name as a variety, synonymising it with *S. concinna*.

Hubendick (1946, p59) noted that "the form has been described as a variant of *S. concinna* Sowerby, on the basis of merely a single specimen". He suggests it is *S. concinna*

and describes it as lying between *S. concinna* and *S. albofasciata* (which is itself a synonym of *S. concinna*).

Allanson (1958) examined the "single, heavily eroded shell" taken from "beach washed specimens" (p163) and notes "a suggestion of a transverse white bar across the internal apex". He suspects it is possibly *S. oculus* but without the actual body and because of the heavily eroded shell, he wrote it is difficult to assign and thus disregards it as a variety or species.

All of Turton's shells were found on the beach at Port Alfred and were thus not collected alive or *in situ* (Turton, 1932). As Turton himself only regarded it as a variety and described it from only one beach found specimen, I believe it is a synonym of *S. concinna* as has already been proposed by Chambers and McQuaid, 1994.

(B) *Siphonaria becki* Turton 1932

Turton (1932, p10) described *S. becki* (12 x14 x3.5mm) after examining three specimens and was convinced of its species status, emphasizing it is "nearly flat and oval". He states that Tomlin agreed with him that this was a new species. The identity of the material shown in his Plate II, figure 81 is inconclusive and could be either *S. oculus* (because of his "nearly flat" description) or *S. concinna* (because of the pointy margin in the figure).

Hubendick (1946, p10) examined four specimens from Port Natal. He commented that "this form has been described on the basis of merely 3 specimens" and it resembled (a) an extreme form of *S. concinna* and (b) was very depressed or flattened. No photograph is shown.

Allanson (1958, p116) examined the type material and concluded that it was *S. oculus*. From personal examination of multiple *S. oculus* individuals, the flatness of the shell is very noticeable in many cases, so I would consider this to be *S. oculus* and propose that the specific name S. *becki* is a synonym of *S. oculus*.

(C) *Siphonaria kowiensis* Turton 1932

Turton (1932) describes this species from one eroded specimen from Port Alfred as having no ribs and being smooth and light brown both inside and outside with a small size, 13x10x5mm. His figure (Plate II, no 84) reflects the severe erosion of the shell, and most closely resembles a dark form of *S. nigerrima*.

Allanson (1958, p159) says the type is "heavily eroded and pitted, and it is difficult if not impossible to assign the specimen either to a new species or an existing one". He suggests it is a small *S. capensis*.

E.T. Reid obtained photographs of the unique holotype shell of *S. kowiensis* from the Oxford Museum and considered this a valid species. However, without examining the type material it is impossible for me to draw a firm conclusion. In any case, the severe erosion and solitary nature of this specimen make it extremely dubious that any name should be attached to it, let alone a new specific name being assigned. I propose the

name *S. kowiensis* should be regarded as a *nomen dubium* rather than *taxon inquirendum* as it presently is in MolluscaBase. It is currently not listed by Catalogue of Life.

(D) *Siphonaria pallida* Allanson, 1958

This was described by Allanson (1958) on the basis of four specimens from Langebaan Lagoon as a variety, *S. aspera* var *pallida,* and not as a separate species, on the grounds of their pale shell colour. He describes the genitalia as being the same as *S. aspera* (now synonymized with *S. serrata*: see Herbert & Warén 1999), except for a smaller accessory organ. The type specimens that I have examined show weakly developed spines on the radial ribs and the siphon is clearly defined by two ribs. MolluscaBase lists *S. aspera pallida* as being accepted as *S. pallida*, but lists *S. pallida* as taxon inquirendum. I would consider it a colour variant of *S. serrata*, and recommend that the entry of *S. pallida* as *taxon inquirendum* in MolluscaBase should be changed, and that it should be formally synonymized under *S. serrata*.

(E) *Siphonaria natalensis* Krauss, 1848

From my examination of Krauss's figure (1848, Plate/Tab 4, No. 6) in the original description of *S. natalensis* (10.5 x 7.5 x 3 mm) it is very likely to be a young specimen of either *S. serrata* or *S. concinna* (Krauss 1848). Hubendick (1946) examined three specimens from Port Natal, and the photograph he provides (Plate V, Fig. 18) resembles a young *S. concinna*.

Allanson (1958) places *S. natalensis* as a synonym of *S. aspera* (now called *S. serrata*) and writes that "the anatomy of the distal genitalia of *S. natalensis* agrees in all respects to that of *S. aspera*" (p171) and that the two prominent siphonal ridges mentioned by Krauss (1848) and Hubendick (1946) are present in elevated (taller) *S. aspera* shells.

On the basis of the preponderance of evidence, I support the view that *S. natalensis* is a synonym of *S. serrata*, concurring with its status in MolluscaBase.

(F) *Siphonaria parcicostata* Deshayes, 1863

From the original (17 x 14 x 7mm) Deshayes description (1863, Plate 7 figures 18 and 19) this appears to be *S. tenuicostulata* (Deshayes 1863). Hubendick (1946) examined 10 shells from Mauritius and one from Port Natal. From his photograph on Plate 3 figures 13 to 15 this could be *S. anneae* or *S. tenuicostulata*. Allanson (1958) reports that the South African record is doubtful because, in spite of detailed surveys on the KwaZulu-Natal coast, there are no further records of this species in South Africa.

I cannot comment on the validity of the name without examining the Reunion sample (the type locality) and some Mauritius samples and therefore am unable to confirm or deny the presence of *S. parcicostata* in southern Africa, but consider it unlikely that it occurs there. The name is accepted as valid by MolluscaBase and Catalogue of Life; with Reunion listed as the type (and only) locality for it.

(G) *Siphonaria deflexa* (Helbling, 1779)

This was the earlier name applied to what is now known as the South African species *S. concinna* Sowerby, 1823. In the unpublished and undated manuscript in which he re-described *S. concinna* Sowerby, Reid provided an extremely detailed and meticulous report on the name 'deflexa' from its very first appearance in the literature. The type locality for *S. deflexa* was never specified, nor can the type material be traced. The description of the species is not sufficient to allow identification of any of the South Africa species as *S. deflexa.* The first description of the species (in Born, 1778) describes it as having a ventral central white patch on the shell, like *S. concinna*, but also describes spines on the external ribs, as in *S. serrata*. As a result, Chambers and McQuaid (1994) consider it to be an unidentifiable *nomen dubium*, as did Kilburn and Rippey (1982). Both pairs of authors therefore adopted the name *S. concinna* for South African material, typically with an internal central white patch on the shell. I have followed their view, and also apply the name *S. concinna*. The situation in MolluscaBase is confusing, because *Siphonaria deflexa* is listed as *nomen dubium*, but *Siphonaria (Patellopsis) deflexa* (the same species but with an allocated subgeneric name) is synonymized under *S. concinna*

(H) *Siphonaria variabilis* Krauss, 1848

This species is currently accepted in MolluscaBase as a junior synonym of *S. concinna* Sowerby, 1823, and from an examination of the detailed figures provided by Krauss (1848, Plate IV, Figs 4a,b), I concur. He illustrates a range of specimens of different sizes, and they parallel the variation exhibited by *S. concinna* as it matures.

Conclusions

The right-hand column of Table 2.1 updates the situation for all the species listed to include the changes of names arising from this chapter, bringing the systematics of *Siphonaria* in southern Africa up to date.

I confirm that *S. concinna* and *S. oculus* are distinct species on the grounds of their forming two separate clades based on genetic composition, and the possession of identifiable differences in their shell morphology. I uphold the views that (a) the name *S. concinna* replaces *S. deflexa* as the latter is regarded as a *nomen dubium*; (b) *S. cyaneomaculata* is a juvenile form of *S. concinna* and is synonymised under that name; (c) the varieties *S. concinna albofasciata* and *S. concinna adjacens* do not reflect genuinely distinct infra-specific taxa and are to be subsumed under the specific name *S. concinna*.

The type of *S. carbo* does not resemble any of the South African east coast species and, in line with Chambers & McQuaid (1994), I propose *S. nigerrima* should be the name accepted for the merged *S. nigerrima* and *S. dayi* because of its earlier date and first placement in Smith's (1903) paper of the original description.

Based on my genetic analysis, members of the '*S. nigerrima'* complex formed a series

running up the east coast of South Africa and southern Mozambique. However, given that the type locality of *S. tenuicostulata* overlaps the range of Clades C and D, it is unlikely that this apparent cline will be upheld with further sampling and genetic analyses. All the members of the *S. nigerrima* complex have direct development with crawling larvae and have unique haplotypes at most localities. Within the complex, five clades were distinguishable. Despite these having been united as a single species, *S. nigerrima*, by Teske *et al.* (2007), I consider the agreement between molecular clades and shell morphology in differentiating these clades to be good evidence that they warrant specific status. One of these (Clade B), comprising specimens from Chidenguele in Mozambique, bears a strong resemblance to material of *S. tenuicostulata*, and I consider this name should be applied to that clade and treated as a valid species. A second clade (Clade D), from Durban, was also genetically separable from other clades, and its shells resembled those of specimens previously identified as *S. anneae* but, because this clade was based on a single specimen in my work, I can only tentatively assign the name *S. anneae* to it. Nevertheless, specimens physically resembling it were found between Durban and Cape St Lucia, and inclusion of material from GenBank showed that it formed a genetic clade spanning that range, strengthening the case for resurrection of this specific name.

A third clade (Clade C), represented by a large number of specimens in the northern section of the east coast of South Africa from Jesser's Point to Ponta do Oura, comprises specimens that can securely be allocated to *S. nigerrima* (sensu lato), including a wide range of shell colour from dark blackish-brown through to predominantly pale forms previously referred to as *S. dayi,* which is now synonymized under the name *S. nigerrima*.

Finally, two clades emerged that had no obvious correspondence with known species in the region, and I consider them to be new species. One of these (Clade E) was collected at Mkambati and analysis of the concatenated sequences suggests it is closely related to Clade D (*S. anneae*). Its shell resembles that of the dark form of *S. nigerrima*, but it has a distinct shell different to that of *S. anneae* and it is probably a separate and undescribed species. The other (Clade A) was collected at Tofo, Mozambique, and is distinguishable on both genetic and morphological grounds, with the homogeneity and distinctiveness of its shell patterning creating a secure case for regarding it an undescribed species.

Of earlier species about which there are doubts, I consider *S becki* is a synonym of *S. oculus*; *S. variabilis* a synonym of *S. concinna*; S. *kowiensis* is a *nomen dubium*; *S. natalensis* is a synonym of *S. serrata*; and the status of *S. parcicostata* cannot be resolved but it is unlikely to occur in southern Africa.

More intensive sampling of the southern east coast of Africa will further clarify the situation and more data, ideally in the form of NGS will increase resolution. Nevertheless, based on the evidence emerging from this chapter: (1) *Siphonaria oculus* and *S. concinna* should be considered separate and valid species; (2) the name *S. nigerrima* should replace *S. carbo* to incorporate South African material of *S. nigerrima* in Clade C together with *S. dayi*; (3) shell morphology suggests that two names until

now synonymised with *S. nigerrima*, namely *S. tenuicostulata* and *S. anneae* should be resurrected to accommodate clades that are recognisable genetically. (4) Genetic evidence points to two additional, undescribed species with affinities to *S. nigerrima.*

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Appendix 2.1
Sample names of specimens collected for genetic analyses, specific names initially attached to samples, collection site, latitude and longitude in decimal degrees, and collector. Names preceded by 'Q' could not be named with certainly in provisional assessments, prior to genetic analyses.

Sample name	Species	Site	Latitude	Longitude	Collector
Q181_Mkb	new species?	Mkambati	-31.319353	29.972663	M. Pfaff
Q428_Mkb	new species?	Mkambati	-31.319353	29.972663	M. Pfaff
Q429_Mkb	new species?	Mkambati	-31.319353	29.972663	M. Pfaff
Q182_Dbn	S. anneae?	Durban	-29.875401	31.061651	C.Moloney
nig94_BhN	*S. carbo/nigerrima*	Bhanga Neck	-27.012069	32.865892	J. Harris
nig012_RR	*S. carbo/nigerrima*	Rabbit Rock	-27.035786	32.86031	J. Harris
nig11_RR	*S. carbo/nigerrima*	Rabbit Rock	-27.035786	32.86031	J. Harris
ten9_BhN	*S. carbo/nigerrima*	Bhanga Neck	-27.012069	32.865892	J. Harris
ann092_BhN	*S. carbo/nigerrima*	Bhanga Neck	-27.012069	32.865892	J. Harris
ten88_RR	*S. carbo/nigerrima*	Rabbit Rock	-27.035786	32.86031	J. Harris
ann090_RR	*S. carbo/nigerrima*	Rabbit Rock	-27.035786	32.86031	J. Harris
ten087_RR	*S. carbo/nigerrima*	Rabbit Rock	-27.035786	32.86031	J. Harris
ann091_BhN	*S. carbo/nigerrima*	Bhanga Neck	-27.012069	32.865892	J. Harris
ann89_RR	*S. carbo/nigerrima*	Rabbit Rock	-27.035786	32.86031	J. Harris
ten10_BhN	*S. carbo/nigerrima*	Bhanga Neck	-27.012069	32.865892	J. Harris
nig093_BhN	*S. carbo/nigerrima*	Bhanga Neck	-27.012069	32.865892	J. Harris
Q331_BhN	*S. carbo/nigerrima*	Bhanga Neck	-27.012069	32.865892	J. Harris
Q330_BhN	*S. carbo/nigerrima*	Bhanga Neck	-27.012069	32.865892	J. Harris
Q329_BhN	*S. carbo/nigerrima*	Bhanga Neck	-27.012069	32.865892	J. Harris
Q114_Tofo	new species?	Tofo, Mozambique	-23.850866	35.543631	L. Kemp
Q113_Tofo	new species?	Tofo, Mozambique	-23.850866	35.543631	L. Kemp
Q117_Tofo	new species?	Tofo, Mozambique	-23.850866	35.543631	L. Kemp
Q116_Tofo	new species?	Tofo, Mozambique	-23.850866	35.543631	L. Kemp
Q115_Tofo	new species?	Tofo, Mozambique	-23.850866	35.543631	L. Kemp
Q112_Tofo	new species?	Tofo, Mozambique	-23.850866	35.543631	L. Kemp
Q369_XX	S. tenuicostulata?	Chidinguele, Mozambique	-24.96107	34.187638	G. Branch
Q368_XX	S. tenuicostulata?	Chidinguele, Mozambique	-24.96107	34.187638	G. Branch
oculus160_Dlb	*S. oculus*	Dalebrook	-34.124327	18.45272	P. de Coito
oculus183_RR	*S. oculus*	Rabbit Rock	-27.035786	32.86031	J. Harris
oculus153_Stga	*S. oculus*	Blythedale	-29.373693	31.350506	C.Moloney
oculus118_BhN	*S. oculus*	Bhanga Neck	-27.012069	32.865892	J. Harris
oculus151_BhN	*S. oculus*	Bhanga Neck	-27.012069	32.865892	J. Harris
oculus166_KpA	*S. oculus*	Koppie Alleen	-34.478244	20.513326	C. Moloney
oculus152_Bal	*S. oculus*	Ballito	-29.537047	31.222306	C.Moloney
oculus154_Wvc	*S. oculus*	Wavecrest	-32.584955	28.524231	G. Branch
oculus439_Mkb	*S. oculus*	Mkambati	-31.319353	29.972663	M. Pfaff
oculus174_RR	*S. oculus*	Rabbit Rock	-27.035786	32.86031	J. Harris
oculus155_BoS	*S. oculus*	Brenton-on-Sea	-34.075209	23.027228	P. de Coito
con28_SBR	*S. concinna*	Stilbaai-Rietvlei	-34.370963	21.602625	L. Kemp
con29_SBR	*S. concinna*	Stilbaai-Rietvlei	-34.370963	21.602625	L. Kemp
con33_SBW	*S. concinna*	Stilbaai-West	-34.394704	21.41404	L. Kemp
con32_SBW	*S. concinna*	Stilbaai-West	-34.394704	21.41404	L. Kemp
cap106_Arn	*S. capensis*	Arniston	-34.676053	20.230123	G. Branch
cap67_Bal	*S. capensis*	Ballito	-29.537047	31.222306	C.Moloney

Appendix 2.2

Additional COI sequences obtained from GenBank and incorporated in my analyses. Collection sites not known if unspecified. Where names recorded in GenBank proved incorrect, the correct names are indicated in the right-hand column, in bold.

Genbank Number	Species	Reference	Site	Latitude	Longitude	Correct name
EF418589	*Siphonaria capensis*	Teske *et al.* 2007				***Siphonaria oculus***
EF418591sp	*Siphonaria nigerrima*	Teske *et al.* 2007				***Siphonaria anneae***
EF418592sp	*Siphonaria nigerrima*	Teske *et al.* 2007				*Siphonaria nigerrima*
EF418593sp	*Siphonaria nigerrima*	Teske *et al.* 2007				*Siphonaria nigerrima*
EF418594sp	*Siphonaria nigerrima*	Teske *et al.* 2007				*Siphonaria nigerrima*
EF418595sp	*Siphonaria nigerrima*	Teske *et al.* 2007				*Siphonaria nigerrima*
EF418596sp	*Siphonaria nigerrima*	Teske *et al.* 2007				*Siphonaria nigerrima*
JN603124	*Siphonaria concinna*	Teske *et al.* 2011	Umhlanga Rocks	29°43'40"S	31°05'20"E	***Siphonaria oculus***
JN603125	*Siphonaria concinna*	Teske *et al.* 2011	Lone Rock	28°54'34"S	31°56'14"E	***Siphonaria oculus***
JN603126	*Siphonaria concinna*	Teske *et al.* 2011	Ponta do Ouro	26°50'40"S	32°53'43"E	***Siphonaria oculus***
JN603127	*Siphonaria concinna*	Teske *et al.* 2011	Jesser Point	27°32'39"S	32°40'47"E	***Siphonaria oculus***
JN603128	*Siphonaria concinna*	Teske *et al.* 2011	Zinkwazi Beach	29°16'58"S	31°26'38"E	***Siphonaria oculus***
JN603129	*Siphonaria concinna*	Teske *et al.* 2011	Warner Beach	30°04'43"S	30°52'23"E	***Siphonaria oculus***
JN603130	*Siphonaria concinna*	Teske *et al.* 2011	Ponta do Ouro	26°50'40"S	32°53'43"E	***Siphonaria oculus***
JN603131	*Siphonaria concinna*	Teske *et al.* 2011	Zinkwazi Beach	29°16'58"S	31°26'38"E	***Siphonaria oculus***
JN603132	*Siphonaria concinna*	Teske *et al.* 2011	Jesser Point	27°32'39"S	32°40'47"E	***Siphonaria oculus***
JN603133	*Siphonaria concinna*	Teske *et al.* 2011	Warner Beach	30°04'43"S	30°52'23"E	***Siphonaria oculus***
JN603134	*Siphonaria concinna*	Teske *et al.* 2011	Salt Rock	29°30'17"S	31°14'18"E	***Siphonaria oculus***
JN603135	*Siphonaria concinna*	Teske *et al.* 2011	Mabibi	27°19'57"S	32°45'06"E	***Siphonaria oculus***
JN603136	*Siphonaria concinna*	Teske *et al.* 2011	Cape St Lucia	28°31'15"S	32°24'04"E	***Siphonaria oculus***
JN603137	*Siphonaria concinna*	Teske *et al.* 2011	Mabibi	27°19'57"S	32°45'06"E	***Siphonaria oculus***
JN603139	*Siphonaria concinna*	Teske *et al.* 2011	Cape St Lucia	28°31'15"S	32°24'04"E	***Siphonaria oculus***
JN603140	*Siphonaria concinna*	Teske *et al.* 2011	Salt Rock	29°30'17"S	31°14'18"E	***Siphonaria oculus***
JN603141	*Siphonaria concinna*	Teske *et al.* 2011	Cape St Lucia	28°31'15"S	32°24'04"E	***Siphonaria oculus***
JN603142	*Siphonaria concinna*	Teske *et al.* 2011	Mission Rocks	28°16'41"S	32°29'11"E	***Siphonaria oculus***
JN603143	*Siphonaria concinna*	Teske *et al.* 2011	Ponta do Ouro	26°50'40"S	32°53'43"E	***Siphonaria oculus***
JN603144	*Siphonaria concinna*	Teske *et al.* 2011	Nine Mile Beach	27°22'58"S	32°43'54"E	***Siphonaria oculus***
JN603145	*Siphonaria concinna*	Teske *et al.* 2011	Cape St Lucia	28°31'15"S	32°24'04"E	***Siphonaria oculus***
JN603146	*Siphonaria concinna*	Teske *et al.* 2011	Lone Rock	28°54'34"S	31°56'14"E	***Siphonaria oculus***
JN603147	*Siphonaria concinna*	Teske *et al.* 2011	Ponta do Ouro	26°50'40"S	32°53'43"E	***Siphonaria oculus***
JN603148	*Siphonaria concinna*	Teske *et al.* 2011	Mission Rocks	28°16'41"S	32°29'11"E	***Siphonaria oculus***
JN603149	*Siphonaria concinna*	Teske *et al.* 2011	Cape St Lucia	28°31'15"S	32°24'04"E	***Siphonaria oculus***
JN603150	*Siphonaria concinna*	Teske *et al.* 2011	Lone Rock	28°54'34"S	31°56'14"E	***Siphonaria oculus***
JN603151	*Siphonaria concinna*	Teske *et al.* 2011	Ponta do Ouro	26°50'40"S	32°53'43"E	***Siphonaria oculus***
JN603152	*Siphonaria concinna*	Teske *et al.* 2011	Warner Beach	30°04'43"S	30°52'23"E	***Siphonaria oculus***
JN603153	*Siphonaria concinna*	Teske *et al.* 2011	Nine Mile Beach	27°22'58"S	32°43'54"E	***Siphonaria oculus***
JN603154	*Siphonaria concinna*	Teske *et al.* 2011	Cape St Lucia	28°31'15"S	32°24'04"E	***Siphonaria oculus***
JN603155	*Siphonaria concinna*	Teske *et al.* 2011	Mission Rocks	28°16'41"S	32°29'11"E	***Siphonaria oculus***
JN603156	*Siphonaria concinna*	Teske *et al.* 2011	Zinkwazi Beach	29°16'58"S	31°26'38"E	*Siphonaria concinna*
JN603157	*Siphonaria concinna*	Teske *et al.* 2011	Blythedale Beach	29°22'27"S	31°21'03"E	*Siphonaria concinna*
JN603158	*Siphonaria concinna*	Teske *et al.* 2011	Blythedale Beach	29°22'27"S	31°21'03"E	*Siphonaria concinna*
JN603159	*Siphonaria concinna*	Teske *et al.* 2011	Lone Rock	28°54'34"S	31°56'14"E	*Siphonaria concinna*
JN603160	*Siphonaria concinna*	Teske *et al.* 2011	Lone Rock	28°54'34"S	31°56'14"E	*Siphonaria concinna*
JN603161	*Siphonaria concinna*	Teske *et al.* 2011	Lone Rock	28°54'34"S	31°56'14"E	*Siphonaria concinna*
JN603162	*Siphonaria concinna*	Teske *et al.* 2011	Umhlanga Rocks	29°43'40"S	31°05'20"E	*Siphonaria concinna*
JN603163	*Siphonaria concinna*	Teske *et al.* 2011	Blythedale Beach	29°22'27"S	31°21'03"E	*Siphonaria concinna*
JN603164	*Siphonaria concinna*	Teske *et al.* 2011	Umhlanga Rocks	29°43'40"S	31°05'20"E	*Siphonaria concinna*
JN603165	*Siphonaria concinna*	Teske *et al.* 2011	Blythedale Beach	29°22'27"S	31°21'03"E	*Siphonaria concinna*
JN603166	*Siphonaria concinna*	Teske *et al.* 2011	Blythedale Beach	29°22'27"S	31°21'03"E	*Siphonaria concinna*
JN603168	*Siphonaria nigerrima*	Teske *et al.* 2011				*Siphonaria nigerrima*
The sequences below were incorporated into the haplotype analysis:						
JN603169	*Siphonaria nigerrima*	Teske *et al.* 2011	Nine Mile Beach	-27.44253	32.69886	*Siphonaria nigerrima*
JN603170	*Siphonaria nigerrima*	Teske *et al.* 2011	Mabibi	-27.332683	32.751775	*Siphonaria nigerrima*
JN603171	*Siphonaria nigerrima*	Teske *et al.* 2011	Jesser Point	-27.540036	32.680016	*Siphonaria nigerrima*
JN603172	*Siphonaria nigerrima*	Teske *et al.* 2011	Mabibi	-27.332683	32.751775	*Siphonaria nigerrima*
JN603173	*Siphonaria nigerrima*	Teske *et al.* 2011	Mabibi	-27.332683	32.751775	*Siphonaria nigerrima*
JN603174	*Siphonaria nigerrima*	Teske *et al.* 2011	Ponta do Ouro	-26.84397	32.894497	*Siphonaria nigerrima*
JN603175	*Siphonaria nigerrima*	Teske *et al.* 2011	Mission Rocks	-28.278333	32.486389	***Siphonaria anneae***
JN603177	*Siphonaria nigerrima*	Teske *et al.* 2011	Zinkwazi	-29.293971	31.45234	***Siphonaria anneae***
JN603178	*Siphonaria nigerrima*	Teske *et al.* 2011	Blythedale	-29.373693	31.350506	***Siphonaria anneae***

Appendix 2.3

Forward (F) and reverse (R) nucleotide sequences of polymerase chain reaction (PCR) primers used to amplify regions of the mitochondrial 16S and COI genes.

Gene	Primer name	Primer sequence (5' to 3')		Reference
large ribosomal subunit 16S	LR-N-13398 (alias 16Sar)(F)	5' cgcctgtttaacaaaaacat 3'	500-650 bp fragment	Simon *et al.*, 1994
	LR-J-12887 (alias 16Sbr or 16Sb)(R)	5' ccggtctgaactcagatcacgt 3'		Simon *et al.*, 1994
Mitochondrial cytochrome *c* oxidase subunit 1 (COI)	LCO1490 (F)	5'-ggtcaacaaatcataaagatattgg-3'	710 bp fragment	Folmer *et al.*, 1994
	HCO2198 (alias H7) (R)	5'-taaacttcagggtgaccaaaaaatca-3'		Folmer *et al.*, 1994

Appendix 2.4

MCMC settings and convergence diagnostics for multiple independent runs in MrBayes. Convergence Diagnostic = CD; average Standard Deviation of Split Frequencies = average SDSF; average Estimated Sample Size = average ESS; Average Potential Scale Reduction Factor = average PSRF. Runs highlighted in bold indicate excellent t race quality.

combined 16s and CO1 dataset			
Run ID	5x2_T	5x2_T2	5x2_T3
run number	**run 1,** run 2	**run 1, run2**	run 1, **run2**
seed	635581044	640605952	298855976
number of generations	40 000 000	40 000 000	40 000 000
sampling frequency	10 000	10 000	10 000
temperature	0.07	0.07	0.07
number of chains	4	4	4
burnin fraction	0.25	0.25	0.25
model: non-coding + coding	16s = HKY+G; CO1 = HKY+I	16s = HKY+G; CO1 = HKY+I	16s = HKY+G; CO1 = HKY+I
acceptance rates	17-51% (both runs)	17-51% (both runs)	17-51% (both runs)
chain swapping %	62-66% (both runs)	64-66% (both runs)	62-66% (both runs)
number of trees produced	4001	4001	4001
number of trees sampled	3001	3001	3001
CD average SDSF	0.0071	0.0044	0.0075
CD average ESS	3000	2500-3000	2700-3000
CD average PSRF	1.000	1.000	1.000
trace quality	run2: bad trace, bimodal AGCT	good (both runs)	run1: bad trace; bimodal AGCT
CO1 only dataset			
Run ID	7x2	8x2	8x2rpt1
run number	**run 1, run 2**	**run 1, run 2**	**run 1, run 2**
seed	49715028	1733028571	1660801218
number of generations	40 000 000	40 000 000	40 000 000
sampling frequency	10 000	10 000	10 000
temperature	0.05	0.05	0.05
number of chains	4	8	8
burnin fraction	0.25	0.25	0.25
model: codon with 3 partitions	1=K80+G; 2=F81; 3=HKY+I	1=K80+G, 2=F81, 3=HKY+I	1=K80+G, 2=F81, 3=HKY+I
acceptance rates	15-71% (both runs)	19-73% (both runs)	19-73% (both runs)
chain swapping %	52-55% (both runs)	3-57% (both runs)	3-57% (both runs)
number of trees produced	4001	4001	4001
number of trees sampled	6002	6002	6002
CD average SDSF	0.006	0.004909	0.004943
CD average ESS	1618-3000	2500-3000	2500-3000
CD average PSRF	1.000	1.000	1.000
trace quality	good (both runs)	good (both runs)	good (both runs)

Chapter 3
Siphonaria compressa, South Africa's rarest endemic mollusc: one species or two?

ABSTRACT

Siphonaria compressa was first described by Allanson (1958) from specimens found in Langebaan Lagoon on the West Coast of South Africa, where it is associated with the seagrass *Zostera capensis*. It has been listed as 'rare and endangered', partly on the assumption that it is endemic to Langebaan Lagoon, but also because its populations undergo periodic collapses associated with environmental changes. In 2005 a population of what was considered the same species was recorded in Knysna Lagoon on the South Coast. I undertook analyses of 16S and COI genes of the two populations to determine their genetic relatedness and assess whether they are conspecific. This revealed that the two populations have no shared haplotypes and are clearly diverged; IMa2 estimates indicate there has been no migration between these populations and the split between them occurred around 730 000 years ago. The Langebaan population has more molecular diversity than does that of Knysna and a number of lines of evidence all point towards recently expanding populations at both sites. Multivariate analyses of 13 morphometric variables from 73 individuals using Analysis of Covariance and Canonical Discriminant Analysis reveals that the means for all variables measured are significantly different and clear differences in shell shape, size and weight occur between the two populations. They can be separated visually and on the basis of the shell height/shell length (SH/SL) ratio. Canonical Discriminant Analysis established that one canonical variable (Canonical Correlation = 0.95) was significant. I thus recognise two geographically delimited species. In an ensuing publication I will describe and name this new species, but for this thesis I distinguish it as *Siphonaria* nov. sp. 3. The fact that the populations are sufficiently distinct genetically to be considered separate species has important ramifications for conservation. In particular, confronted with periodic population collapses of both populations, it is no longer a viable option to consider population transfers as a means of restoring populations.

3.1 INTRODUCTION

Siphonaria compressa Allanson, 1958 – the smallest of the southern African Siphonariidae, with a shell length of up to 4 mm – is an endemic, critically endangered, pulmonate marine mollusc found only on Cape eelgrass, *Zostera capensis* (Fig. 3.1) (Herbert 1998). In its restriction to sheltered lagoonal habitats, narrow range of salinity tolerance (Wilson *et al*. 2009) and strong dependence on the eelgrass on which it lives (Angel *et al.* 2006), it has a different pattern of distribution compared to all other species of *Siphonaria* that occur in this region. It is currently regarded as being found only in Langebaan Lagoon and Knysna Estuary. As such, is it South Africa's most endangered marine mollusc and is classed as 'critically endangered' (Herbert 1999).

Figure 3.1 Top: Cape eelgrass bed at Klein Oesterwal, Langebaan Lagoon, Western Cape, South Africa. Bottom: *Siphonaria* nov.sp. 3 from Knysna Estuary on Cape eelgrass (*Zostera capensis*) (Photo: GM Branch).

Seagrasses such as *Zostera capensis* are found in shallow estuarine and coastal water throughout the world and are sensitive to temperature, turbidity and salinity stress leading to quick die-off if conditions become unfavourable; they are under threat globally (Orth *et al.* 2006). In South Africa *Z. capensis* is found in all permanently open estuaries between the Olifants Estuary on the west coast of South Africa and the eastern border of the country, extending up through Mozambique to the Western Indian Ocean (Richmond 2011, Adams 2016).

There has been a 38% loss of seagrass in Langebaan Lagoon between 1960 and 2007, with monitoring between 1983 and 2009 indicating that invertebrate abundance and species richness declined significantly with the decline in *Zostera* (Pillay *et al.* 2010). Elevated temperatures increase the smothering of *Z. capensis* by epiphytic microalgal growth, but *Siphonaria compressa* has a beneficial effect on the seagrass by grazing on the epiphytes and keeping blades free of fouling (Waspe 2015).

A species such as *S. compressa* with both a narrow salinity tolerance and an obligate dependency on seagrasses is doubly affected by any environmental changes. An apposite example of how this can play out is that of *Lottia alveus,* a gastropod limpet found only on the eelgrass *Zostera marina* on the eastern coast of North America, which became the first marine invertebrate known to have become extinct in historical time (Carlton *et al.* 1991).

In South Africa, *S. compressa* was for years known only from the single extant population in Langebaan Lagoon on the cool-temperate west coast (Herbert 1998, 1999) where drastic fluctuations in population size have been reported (Angel *et al.* 2006). Both Angel *et al.* (2006) and Siebert and Branch (2007) have published on the ecology of *S. compressa* in Langebaan Lagoon and have discussed reasons for its rarity and constrained habitat, which include its restriction to eelgrass, the constraint of the eelgrass to the upper shore by the burrowing activities of the sandprawn *Kraussillichirus kraussi*, and limitations on its penetration up the shore because of its intolerance of desiccation. Within the eelgrass beds at Langebaan the mollusc is generally confined to the lower portion of the beds and is replaced by *Assiminea globulus* higher up (Angel *et al.* 2006). Wilson *et al.* (2009) have elucidated how its low salinity tolerance restricts it to estuaries with a narrow range of salinities.

In 2004, a population of what was considered to be *S. compressa* was discovered in Knysna Lagoon, 650 km from Langebaan (Allanson & Herbert 2005; Allanson & Msizi 2010) (Figure 3.2). Knysna is a more traditional estuary in the sense that it has a riverine input, which predominantly affects the upper reaches, whereas its lower reaches where *S. compressa* occurs are more bay-like, and are tidally dominated and experience relatively constant salinities approximating those of seawater, except during episodic floods. The latter do not occur in Langebaan. Langebaan is classed as an 'estuarine embayment' by Whitfield (2005), placing it within the definition of estuarine systems (Whitfield & Elliot, 2011) but distinguishing it from other estuaries in that it has minimal freshwater input.

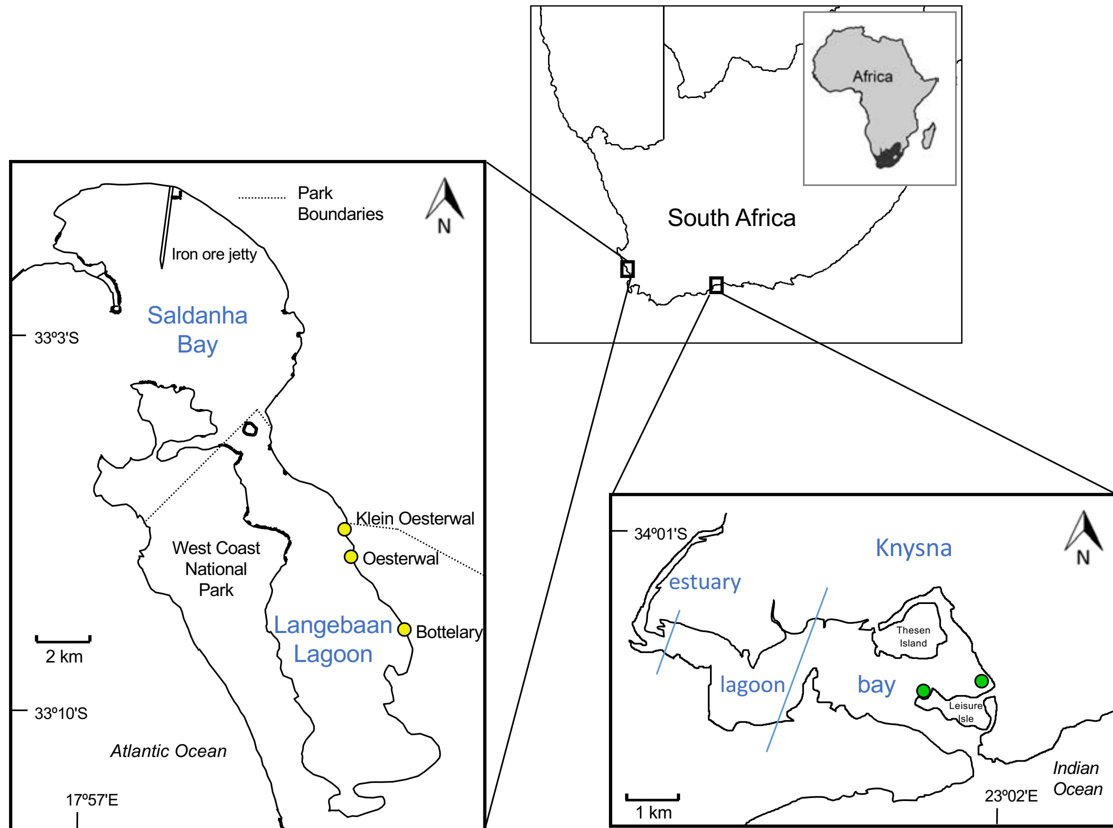

Figure 3.2 Maps of Africa, South Africa and the two sites where populations that have been identified as *S. compressa* have been recorded. Yellow and green circles indicate collection sites.

As referred to above, there is evidence for population fluctuations at Langebaan: Angel *et al.* (2006) recorded expanding and contracting population sizes over a number of years. Periodic severe flooding of the Knysna Lagoon both before and since the discovery of *S. compressa* there would suggest that this population too has undergone repeated expansion and contraction. Severe floods during 22 and 23 December 2004 definitely affected the Bollard Bay population in Knysna, close to one of the sites where I sampled (B. Allanson, *pers. comm.*). Herbert (1999) recorded collecting beach-drift shells, putatively of *S. compressa*, in 1995 at the nearby Keurbooms Estuary 30 km east of Knysna.

To my knowledge there has been no molecular research on the species bar that of Chambers *et al.* (1998), who found within-population variability for *S. compressa* at Langebaan Lagoon to be the highest out of the 12 siphonariids studied with RAPD fingerprinting. As *S. compressa* is a direct developer with the most restricted distribution of all the siphonariids compared, and thus the least dispersive, they found that surprising.

Because the two populations share the same habitat and are morphologically similar in shell shape and size, they have thus far been considered as the same species and the name *S. compressa* applied to both. However, the extreme geographic separation and absence of any intervening populations in estuaries with *Zostera* raise the possibility that they are the remnants of a once wider-spread ancestral population

that has split into sister taxa that have diverged over time due to isolation. Indeed, I hypothesise that is the case, and the central focus of this chapter is on whether the two populations are genetically and morphometrically distinguishable or not.

There are two alternative explanations for the occurrence of *S. compressa* in these two lagoons. The first is that it could have been introduced from one lagoon to the other by vessels travelling between them. Peters *et al.* (2014) have established that many small-vessel harbours share species because of transport by yachts. Alternatively (or additionally) transfers may have been associated with aquaculture activities, as both localities have been active sites for aquaculture, and Haupt *et al.* (2010) have documented the role of oyster aquaculture in such transfers. Either of these avenues would imply that one of the populations is effectively alien and a relatively recent introduction. However, genetic analyses have the potential to determine (a) if the connection between the populations is recent, admitting a human-mediated introduction, or ancient, supporting my hypothesis that the populations are relics of a broader ancestral population; and (b) if recent transportation has taken place, which of the two populations is the source.

Resolution of these issues has considerable implications for conservation, which are explored in the Discussion. There is thus a strong practical application to this research. Designation of species and conservation status (as different Evolutionary Significant Units for management) can be used to inform decisions regarding the declaration of Marine Protected Areas (MPAs) around South Africa (Teske *et al.* 2008, 2011; von der Heyden *et al.* 2014), especially given the hotspot traits of high endemism and high environmental threat to both lagoons. The two sites where these molluscs occur are both in MPAs, but should one become extinct, which is a distinct possibility with the increased unpredictability and extreme nature of weather patterns under global warming and the history of severe flooding in the region, options such as translocation or genetic augmentation will depend on their genetic relatedness.

In summary, there are two aims for this chapter. The first is to quantify the genetic diversity of the two populations of *Siphonaria compressa* and investigate their demographic history, thereby determining the relatedness between the two extant populations found in South Africa. The second is to explore by means of morphometric analysis whether there are differences in shell shape between the two populations, to provide supporting evidence for any differentiation between the two populations.

3.2 MATERIALS AND METHODS

Sample collection

Between 2005 and 2008, ninety *Siphonaria compressa* individuals in total were collected from sites indicated in Figure 3.2 at Langebaan Lagoon (Klein Oesterwal -33.1254, 18.0589; Oesterwal -33.1284, 18.0696; Bottelary -33.1439, 18.0920) and Knysna Estuary (Leisure Isle -34.0608, 23.0515; Bollard Bay -34.0639, 23.0665). All material was immediately preserved in 90-98% ethanol. All material was

collected under permit issued by Environmental Affairs, Department of Agriculture, Forestry and Fisheries, and in accordance with ethical requirements then in force at the University of Cape Town. In view of the record of beach-drift shells of the species at Keurbooms Estuary (-34.02, 23.39) by Herbert (1999), I conducted an extensive search for live or drift material there in March 2008, but failed to detect the species despite the presence of suitable *Z. capensis* habitat.

Laboratory procedures and sequence analysis

Details of the methodology for DNA extraction, choice of markers and DNA amplification and sequencing, as well as sequence assembly, multiple sequence alignment, summary statistics and haplotype construction are outlined in Chapter 2, except for details that depart from those procedures, as outlined here. Base frequencies and pairwise genetic distances for each lagoon and between the two lagoons were calculated (custom R script) for each gene fragment.

Historical demography

(A) Tajima's D and Ramos-Onsins and Rozas' R2
Two neutrality indices, Tajima's D (Tajima 1989) and Ramos-Onsins and Rozas's R2 (Ramos-Onsins & Rozas 2002) were calculated to detect whether the variation found was consistent with increasing or contracting populations.

(B) Mismatch distribution
A nucleotide mismatch distribution providing a histogram of the frequencies of pairwise distances between sequences was calculated to identify trends in population size and to test the null hypothesis of constant population size (Slatkin & Hudson 1991; Rogers and Harpending 1992).

(C) Isolation with Migration Analysis
Isolation with Migration Analysis was implemented in IMa version 2.0 (IMa2) (Hey & Nielsen 2007). This generates posterior probabilities for time and migration parameters for the 'Isolation with Migration' population genetic model (Nielsen & Wakeley 2001) using a Bayesian approach and Markov chain Monte Carlo (MCMC) simulations of gene genealogies. The method is appropriate for estimating divergence time of pairs of closely related populations or species that have recently separated, as well as the presence, direction, and magnitude of migration between them. Two datasets were analysed in M mode in IMa2: COI (657 base pairs), for which the mutation rate is well documented; and the concatenated 16S+COI (1097 base pairs) for 43 Langebaan and 40 Knysna sequences. For each dataset multiple identical independent runs starting with different seeds generated a combined total of 13384 genealogies for the COI dataset and 39461 genealogies for the 16S+COI dataset.

A mutation rate for the COI locus was chosen as 1% per million years (Meyer *et al.* 2005) and for the 16S rate, 0.4% per million years (Teske *et al.* 2007). A combined mutation rate incorporating the proportion of each marker was calculated for the concatenated 16S+COI dataset as the two gene fragments are strictly speaking two portions of the same locus and a single rate is more appropriate.

A mutation rate in the format required by IMa2 of 8,33 x 10^{-6} (equivalent to 0.759% per million years) was calculated as follows: rate for COI portion: 1% per million years = 0.01 x 10^{-6} = 0.000 000 01; 657 COI bp/1097 total bp = 0,5989; 0.5989 x 0.000 000 01= COI rate. Rate for 16S portion: 0.4% per million years = 0.004 x 10^{-6} = 0.000 000 004; 440 16S bp/1097 total bp =0.4011; 0.4011 x 0.000 000 004 = 16S rate. Combined rate: [(0.5989 x 0.000 000 01)+(0.4011 x 0.000 000 004)] x 1097 base pairs = 8.33E-06.

Each run used a geometric heating scheme with 100 chains and heating parameters ha = 0.96 -hb = 0.9. A burnin of 500 000 iterations (based on examination of likelihood/generation plots) was used.

Different prior estimates were initially explored to identify appropriate bounds for the prior distributions of the demographic parameters (Hey 2011). This was followed by test runs to check for adequate MCMC chain mixing (essential for generating independent genealogies), heating, adequate swap and update acceptance rates and ESS values (effective sample size or number of independent samples) of at least 200. Items checked were (1) that the runs were mixing well and that the MCMC simulation explored the state space (with no trends evident in plots), (2) effective sample size estimates were adequate, (3) acceptance rates were good and (4) that the two sets of sampled genealogies of SET1 and SET2 in the output were similar. The burnin period was modified to improve peak formation in demographic parameter scatterplot results.

From an initial run of the data, it was established that there was no migration between the two populations and subsequent prior files were modified to reflect this. Multiple independent runs of both datasets with different start seeds gave similar results, providing evidence that the estimates made for the parameters were robust. The peaks of the estimated distributions are taken as the estimates of the parameters.

(D) Bayesian skyline plots

Bayesian skyline plots (Drummond *et al.* 2005), constructed with BEAST v1.8.0 (Drummond *et al.* 2012) were used to estimate trends in effective population sizes through evolutionary time. Topali v2 (Milne *et al.* 2009) was used for evolutionary model selection with HKY+G (Hasegawa *et al.* 1985) being selected for all data partitions and combinations.

The same mutation rates were used as for IMa2 above, but were adjusted so that the clock rate used for the skyline plots provided an X-axis in units of years.
The rate used for COI was 5.0 E-9, and for the combined 16S+COI data the rate was set at 3.79 E-9, and both were run for 50 million generations with a 10% burnin.

Empirical base frequencies and a strict clock model (a global clock rate without variation among lineages) were specified. Final runs were repeated three times and the logoutput files joined with Tracer v1.6 (Rambaut *et al*. 2014) (after removal of burnin) to check traces and assess convergence, and that ESS values were above 200; their trees (13501) were combined in LogCombiner v1.8 (a component of BEAST 1.8.0) and Skyline plots were constructed in Tracer v1.6.

Morphometric analyses

I compared the shell morphometrics of specimens from the two populations using a combination of (1) linear measurements, (2) indices derived from ratios, (3) regressions of variables and (4) multivariate analysis – all approaches adopted by Uba (2021) in comparing the morphometrics of two congeneric bivalves.

The data set contained observations on 73 specimens: 30 from Langebaan and 43 from Knysna. All shells used in the examination of morphometric variation were in good condition with limited or no erosion. Washed and dried shells were weighed to four significant places on a Mettler AE100 balance. Shells were photographed with a Nikon SMZ 1500, imported into ImageJ (Abramoff *et al*. 2004, Schneider *et al.* 2012) and the following measurements taken (Figure 3.3): shell length (SL, greatest distance between anterior and posterior end), shell height (SH, greatest vertical distance from the top of the shell to the plane of the aperture), shell width (SW, greatest distance perpendicular to the anterior-posterior axis), apex length (AL, distance from apex to posterior shell margin, apex width (AW, distance from apex to the maximum protrusion of the shell width on the left) and apex height (AH, distance from the apex to the plane of the aperture). The following ratios were also calculated: SH/SL, SH/SW, SW/SL, AW/SW, AL/SL and AH/SH. Note that the point of the apex from which apex length/width/height is measured differs from that from which shell height is measured due to the curvature of the shell, and that it was possible to have a negative value for apex length if the apex protruded beyond the posterior of the shell margin.
However, there was only one instance of this (one specimen at Langebaan), and it was excluded from the analyses.

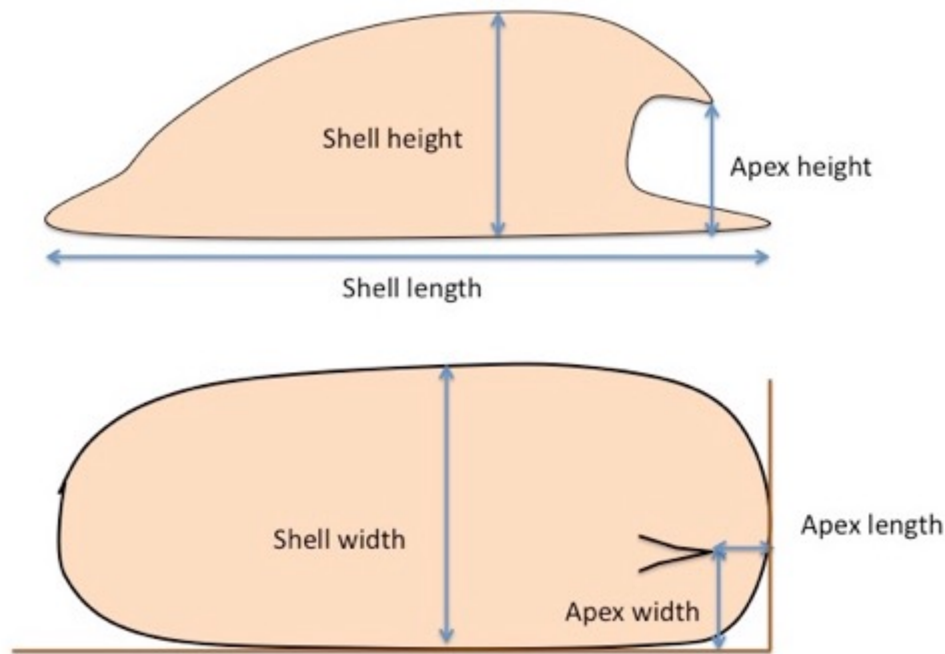

Figure 3.3 Schematic shell in lateral and dorsal view indicating how measurements were taken.

(A) Univariate and bivariate (regression) analysis

All univariate analyses were run with R Version 3.5 (R Core Team 2016) in RStudio 1.1.447 (RStudio Team 2016). Prior to performing any statistical tests, both raw and log10 transformed data for the 13 characters measured for shells at the two sites were checked for normality using quantile-quantile (qq) plots: no variables were distinctly non-normal, with most of the dots falling along the qq lines. The log10 transformed data were a better fit. In addition, sample size was quite large ($n \geq 30$).

To test the null hypothesis of equal means for each variable between populations, t-tests were performed. The shell height and mass data had variances differing by more than a factor of 5, which necessitated the use of a Welch Two Sample t-test which does not assume similar variances. For all other variables the two populations were homogeneous enough to run a normal Student's t-test which assumes equal variances.

Linear regression analysis was performed to compare the shell length and mass variables and an Analysis of Covariance (ANCOVA) was used to test for the significance of the differences between the slopes and intercepts of the two regression lines.

(B) Multivariate statistical analysis

Canonical Discriminant Analysis (JMP Version 15.2) (SAS Institute Inc.) was used to test the null hypothesis that the two populations have the same multivariate mean. This technique derives linear combinations of variables, i.e., Canonical Variates (CVs), such that the correlations between the CVs and group membership and the ratio of between- to within-group variance are maximized (Krzanowski, 1990). Correlation between the original variables and the derived CVs, as well as the patterns of loading for the original measurements, allow reification of the CVs in terms of shape and size differences among

groups. The analysis was applied to the same data set as that described above adjusted to remove samples for which there were no shell weights. The truncated data matrix consisted of observations on 30 specimens from Langebaan and 32 from Knysna (Appendix 3.1). The assumption of normality was previously established. To ensure the relationships between the variables were linear the measurements were log10 transformed before analysis.

3.3 RESULTS

Final DNA datasets constructed were a 16S dataset of 43 sequences 440 base pairs in length, and a COI dataset of 43 sequences 661 base pairs in length. The 16S and the corresponding COI sequences from the same individuals were combined to give a trimmed concatenated dataset (43 sequences x 1097 base pairs combined dataset). The analyses produced a total of 83 new sequences all of which will be submitted to GenBank. The morphometric analysis generated a data set of 13 variables for 73 individuals (Appendix 3.2).

The shells of all specimens not damaged during DNA extraction or morphometrics analyses will be deposited with the KwaZulu-Natal Museum, South Africa, where the Chambers & McQuaid (1994) voucher samples mentioned in the previous chapter are currently stored.

Summary statistics

Table 3.1a summarises the genetic variation, base frequencies and tests of population expansion for the two populations. The COI segment had greater variation (haplotype count = 39; nucleotide diversity = 0.0103) than the 16S segment (haplotype count = 9; nucleotide diversity = 0.0032).
The average genetic distance (p-distances) calculated for the coding COI portion for within the lagoons individually shows that Langebaan (0.0053) had twice as much variation as Knysna (0.0026). Similarly, the p-distances for the combined 16S+COI length were 0.0035 for Langebaan and 0.0019 for Knysna (Table 3.1b).

Table 3.1 Genetic diversity indices and population statistics for the two populations of *Siphonaria compressa* at Langebaan and Knysna for (a) 16S and CO1 genes for both populations separately and combined, and (b) average genetic distance between populations.

(a)

Population statistics

	16s (440bp)			CO1 (661bp)			16s+CO1 (1097bp)		
	both sites	**Langebaan**	**Knysna**	**both sites**	**Langebaan**	**Knysna**	**both sites**	**Langebaan**	**Knysna**
number of sequences	90	45	45	83	43	40	83	43	40
haplotypes	9	4	5	39	22	17	42	23	19
segregating sites	9	4	5	36	21	17	45	25	22
nucleotide diversity	0.0032	0.00053	0.00082	0.0103	0.0053	0.0026	0.0077	0.0035	0.0019
Tajima's D	-0.693	-1.789	-1.7736	-0.1731	-0.9625	-1.8788	-0.3166	-1.232	-2.0227
TD's p value	0.4883	0.0736	0.0761	0.8626	0.3358	0.0603	0.7516	0.218	0.0431
R2 (x10000)	0.0708	0.0423	0.0475	0.0913	0.0752	0.0481	0.087	0.0672	0.0444
R2 p value	0.2799	0.0012	0.011	0.477	0.1713	0.006	0.4487	0.1071	0.0004
GC content	0.362	0.3624	0.3617	0.3609	0.3573	0.3599	0.3615	0.3593	0.361
base frequency a	0.3196	0.3184	0.3208	0.2367	0.2371	0.2391	0.2696	0.2696	0.2716
base frequency c	0.2025	0.2014	0.2036	0.1572	0.1528	0.1577	0.1753	0.1722	0.1761
base frequency g	0.1595	0.1609	0.1581	0.2037	0.2044	0.2023	0.1862	0.187	0.1849
base frequency t	0.3184	0.3192	0.3175	0.4024	0.4056	0.401	0.3689	0.3711	0.3675

(b)

Average genetic distance between populations

	Mean	SD	SE
16S			
Between populations	0.0058	0.0013	2.80E-05
Within Knysna	0.0008	0.0011	2.34E-05
Within Langebaan	0.0005	0.0012	2.78E-05
COI			
Between populations	0.0168	0.0028	6.68E-05
Within Knysna	0.0026	0.0020	4.89E-05
Within Langebaan	0.0053	0.0032	7.41E-05
16S+COI			
Between populations	0.0126	0.0017	4.18E-05
Within Knysna	0.0019	0.0013	3.36E-05
Within Langebaan	0.0035	0.0020	4.56E-05

Haplotype diversity

Parsimony haplotype diagrams constructed for each of the three datasets (16S, COI, 16S+COI) (Figure 3.4, Table 3.1a) show (a) fewer haplotypes present in the 16S portion, and (b) that none of the haplotypes is shared between the two localities for either of the gene segments.

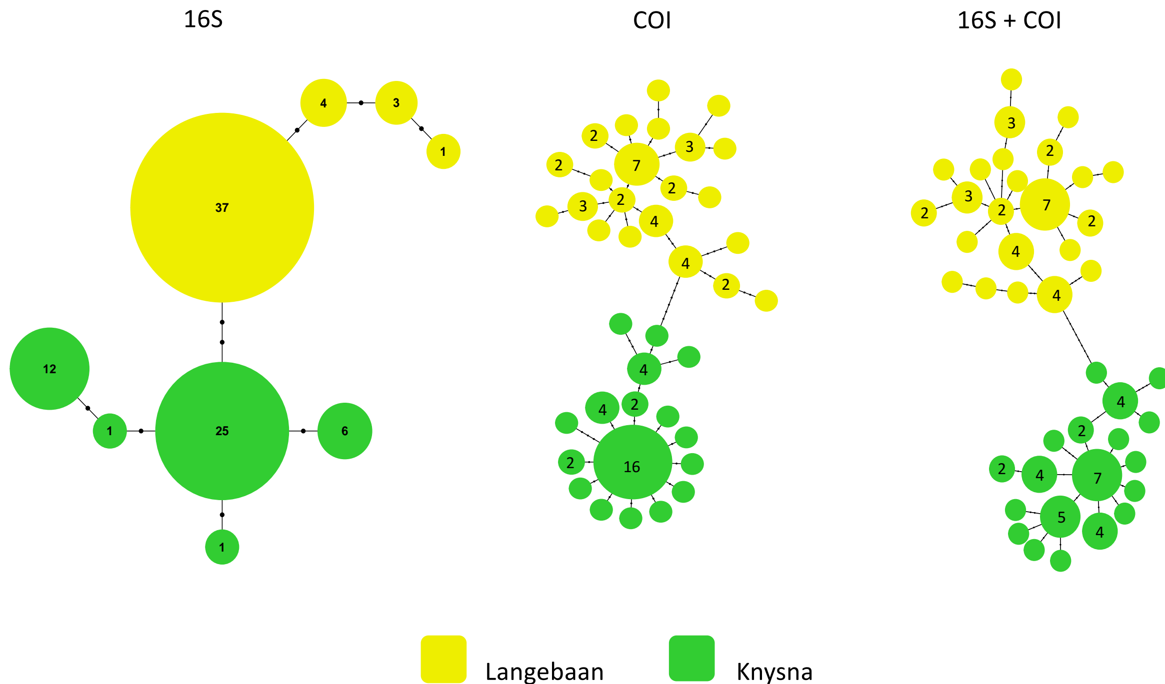

Figure 3.4 Haplotype parsimony diagram for *Siphonaria compressa* 16S, CO1 and 16S+COI mtDNA sequence data. Each circle represents a different haplotype with the numbers within circles indicating the number of individuals with that haplotype. Circles with no numbers indicate a single individual. Haplotypes unique to Langebaan are coloured yellow and those to Knysna, green. Unsampled haplotypes are represented by small dots.

Historical demography

(A) Tajima's D and R2 neutrality indices (Table 3.1a) were calculated to detect any recent population expansion, a deviation from the assumption of neutrality. Tajima's D for Knysna (-2.023, p=0.043) and Langebaan (-1.232, p=0.22) were calculated for 16S+COI combined; both were negative. Values of Tajima's D are significant at 0.05 if they are either >2 or <-2. Knysna's combined R2 value (0.044, p=0.0004) was lower than that of Langebaan (0.067, p=0.107).

(B) Mismatch distribution

Unimodal mismatch distribution diagrams for each lagoon (Figure 3.5) exhibit a single smooth peak indicating expanding populations in both lagoons and corroborating the Tajima's D and R2 indices.

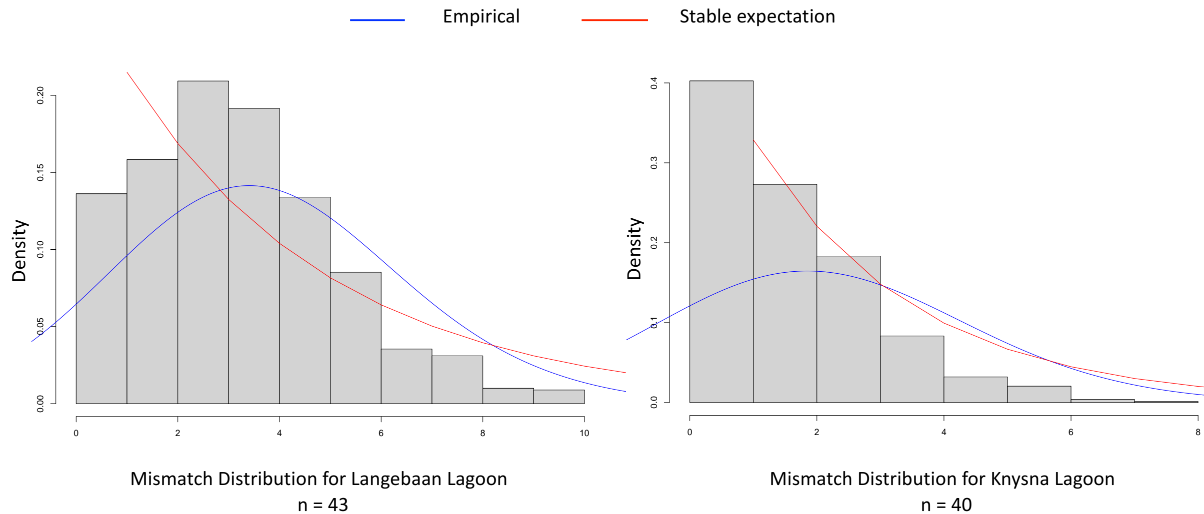

Figure 3.5 Mismatch distribution diagrams for both lagoons from the combined 16S+CO1 dataset. Note the different scales on the axes.

(C) Isolation with Migration Analysis

Table 3.2 summarises the IMa2 results. Demographic estimates were similar for the two datasets. It was established from an initial run that no migration occurred between the two populations. The time of divergence between the two lineages is estimated at approximately 730 000 years ago with Highest Posterior Density (HPD) intervals ranging from ~300 000 to ~1.3 million years. Effective population size estimates for Langebaan and Knysna were 1.3 million (HPD ~700 000 to ~2.5 million) and 1 million respectively (HPD ~500 000 to 2 million). The HPD intervals of the effective population sizes for the concatenated dataset were narrower than those of the CO1 dataset. The ancestral population is estimated to have been about 340 000 individuals (HPD zero to 45 million). All HPD intervals overlapped.

The 95% HPD (highest posterior density) interval is similar to a confidence interval, and is the shortest span (on the x-axis) that contains 95% of the posterior probability. It is bounded by the HPD95Lo and the HPD95Hi. The HPD95Lo and the HPD95Hi for both datasets overlap: any differences may be as a result of inaccuracies in the original mutation rates selected.

Appendix 3.2 provides details of parameters used and output for each of the runs for the two datasets.

Table 3.2 Summary table of divergence times, effective population sizes (number of females) in demographic units and migration rates of the two populations of extant *Siphonaria compressa* estimated with IMa2 for the COI and the 16S and COI combined datasets. M0>M1 is the number of individuals received by Population 2 per generation; M1>M0 is the number of migrants received by Population 1 per generation. * Values in brackets are the 95% Highest Posterior Density Intervals.

	Population 1 (Langebaan) sample size	Population 2 (Knysna) sample size	T (divergence time in years)	N0 (Langebaan population size)	N1 (Knysna population size)	N2 (ancestor population size)	M0>M1 to Knysna	M1>M0 to Langebaan
COI	N = 43	N = 40	760,845 (309,361-1,359,589)*	1,412,671 (779,110-2,491,438)*	1,098,744 (567,922-2,023,402)*	356,736 (0-43,864,155)*	0	0
16s + COI	N = 43	N = 40	695,887 (298,923-1,211,299)*	1,187,739 (677,529-2,050,595)*	1,003,164 (533,470-1,811,997)*	330,136 (0-46,669,228)*	0	0

(D) Bayesian skyline plots

Bayesian skyline plots (Figure 3.6) of the effective population sizes through time for the two *S. compressa* populations are based on the haplotype genealogies of the concatenated 16S+COI dataset and show that the most recent common ancestor (MRCA) where the extant haplotypes coalesce for the Langebaan population (approximately 300,000 years ago) is older than that of the Knysna population (approximately 150,000 years ago). Both populations show a gradual increase in population size up to the present. For the extant populations this predicts a median of 3 million for Langebaan and just over 5 million individuals for Knysna with confidence intervals of 400,000 to 120 million and 650,000 to 140 million respectively. The CO1 dataset estimates similar values.

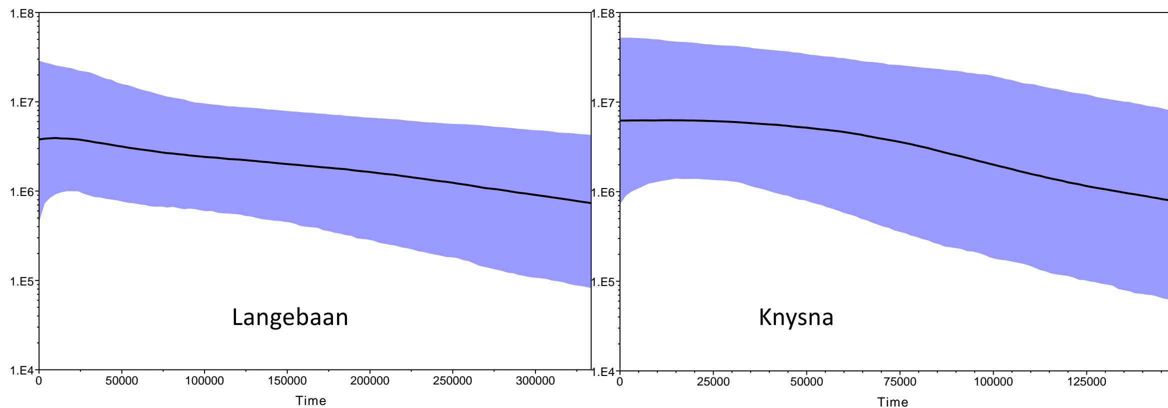

Figure 3.6 Skyline plots for Langebaan and Knysna Lagoons from the combined 16S+CO1 dataset, produced by joining the results of three independent runs of BEAST in LogCombiner. Dates are years before present.

Morphometric analyses

(A) Univariate and bivariate (regression) analysis

There was clear statistical differentiation between the two populations, for all seven absolute morphometric variables measured (Table 3.3). In particular, shell length and mass differed, with Langebaan individuals tending to be longer (range 2.55 – 5.35mm, median 3.67mm) and heavier (range 1.1 – 9.8mg, median 3.3mg) than those from Knysna (range 2.60 – 4.26mm, median 3.30mm; range 0.4 – 2.5mg, median 1.2mg)(Figure 3.7 a,b). All six ratios between variables except the ratio of apex height to shell height (AH/SH) also showed significant differences between the two populations at $p < 0.001$ (Figure 3.7, Table 3.3).

Table 3.3 Summary of morphometric variables by population: means, ± standard errors of the mean, ranges, t-test values, degrees of freedom, significance values and R^2 values for various pairs of variables.

shell character	Langebaan mean	Langebaan +/-SE	Langebaan range	Knysna mean	Knysna +/-SE	Knysna range	t-test	df	p
		n=30			n=32				
mass (M)(mg)	3.99	0.485	1.1 - 9.8	1.25	0.10	0.4 - 2.5	-5.537	31.55	4.36E-06
		n=30			n=43				
length (SL)(mm)	3.83	0.13	2.55 - 5.35	3.28	0.06	2.60 - 4.26	-4.09	71	0.0001126
width (SW)(mm)	2.07	0.05	1.55 - 2.64	1.69	0.03	1.34 - 2.13	-6.76	71	3.14e-09
height (SH)(mm)	1.81	0.07	1.16 - 2.56	1.06	0.03	0.69 - 1.41	-9.53	36.89	1.75E-11
apex length (AL)(mm)	0.30	0.03	0.06 - 0.65	0.50	0.02	0.22 - 0.79	6.15	71	4.06E-8
apex width (AW)(mm)	0.44	0.02	0.22 - 0.63	0.53	0.01	0.41 - 0.66	4.03	71	0.000138
apex height (AH)(mm)	1.19	0.05	0.61 -1.77	0.72	0.03	0.37 - 1.17	-8.61	71	1.23e-12
Ratios:									
height/length (SH/SL)	0.47	0.00	0.42 - 0.52	0.32	0.00	0.25 - 0.39	-22.59	71	<2.2e-16
height/width (SH/SW)	0.87	0.02	0.73 - 1.07	0.62	0.01	0.45 - 0.74	-13.12	71	<2.2e-16
width/length (SW/SL)	0.55	0.01	0.47 - 0.63	0.52	0.00	0.47 - 0.57	-3.88	71	0.000233
apex width/width (AW/SW)	0.22	0.01	0.10 - 0.32	0.31	0.01	0.22 - 0.40	8.07	71	1.24e-11
apex length/length (AL/SL)	0.08	0.01	0.02 - 0.19	0.15	0.00	0.07 - 0.23	8.78	71	6.08e-13
apex height/height (AH/SH)	0.65	0.01	0.51 - 0.75	0.68	0.01	0.47 - 0.83	1.41	71	0.164

	R2 Langebaan	R2 Knysna
log10mass~log10length	0.91	0.84
height~length	0.95	0.77
height~width	0.87	0.63
width~length	0.91	0.84
mass~width	0.75	0.73
mass~height	0.88	0.77

Whereas the ranges overlapped for most of the 13 morphometric variables (Figs 3.7, 3.8), there was no range overlap for the shell height/shell length (SH/SL) ratio (Figure 3.8a), making it the most diagnostic of the ratios followed by shell height/shell width (SH/SW) (Figure 3.8b). Apex height/shell height (AH/SH)(Figure 3.8f) was the least diagnostic, followed by shell width/shell length (SW/SL) (Figure 3.8c). Apex width/shell width (AW/SW)(Figure 3.8d) and apex length/shell length (AL/SL) (Figure 3.8e) were intermediate. In general, there was more variability in the Langebaan measurements (Table 3.3, Figure 3.8).

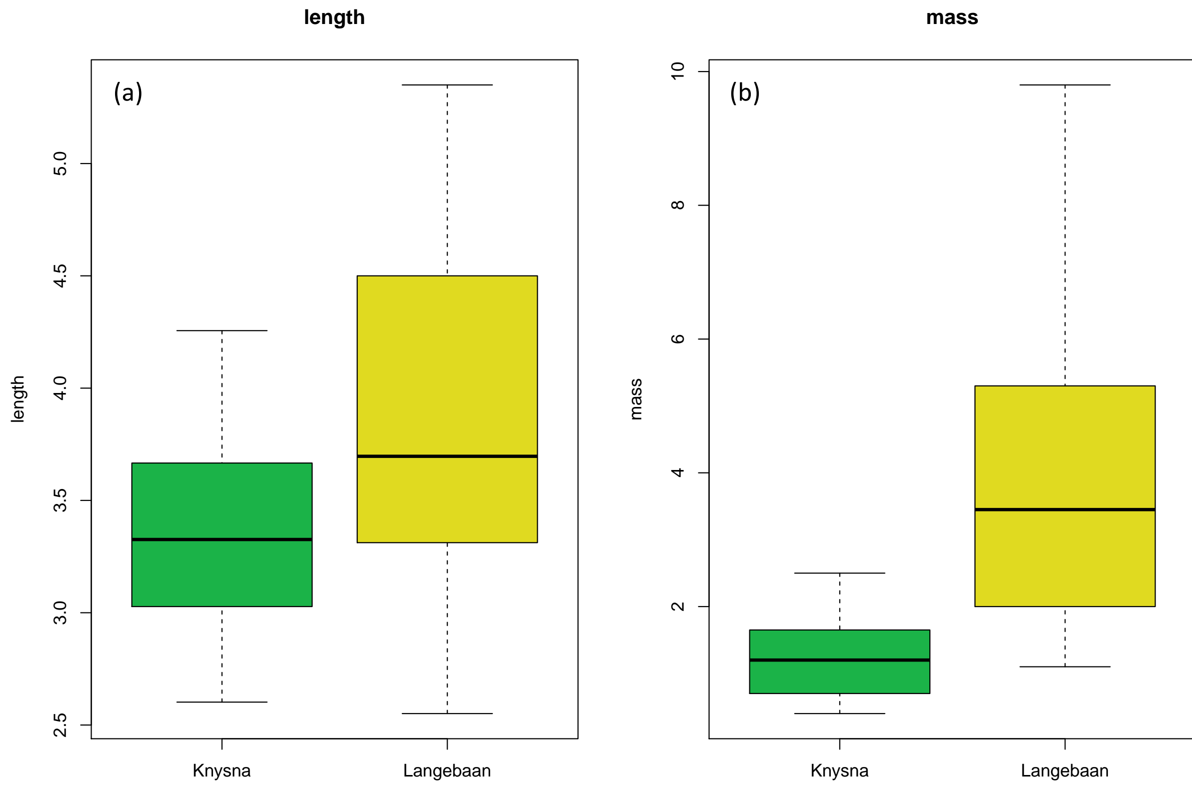

Figure 3.7 Boxplots for the shell length in mm (a) and shell mass in mg (b) data for the two lagoons.

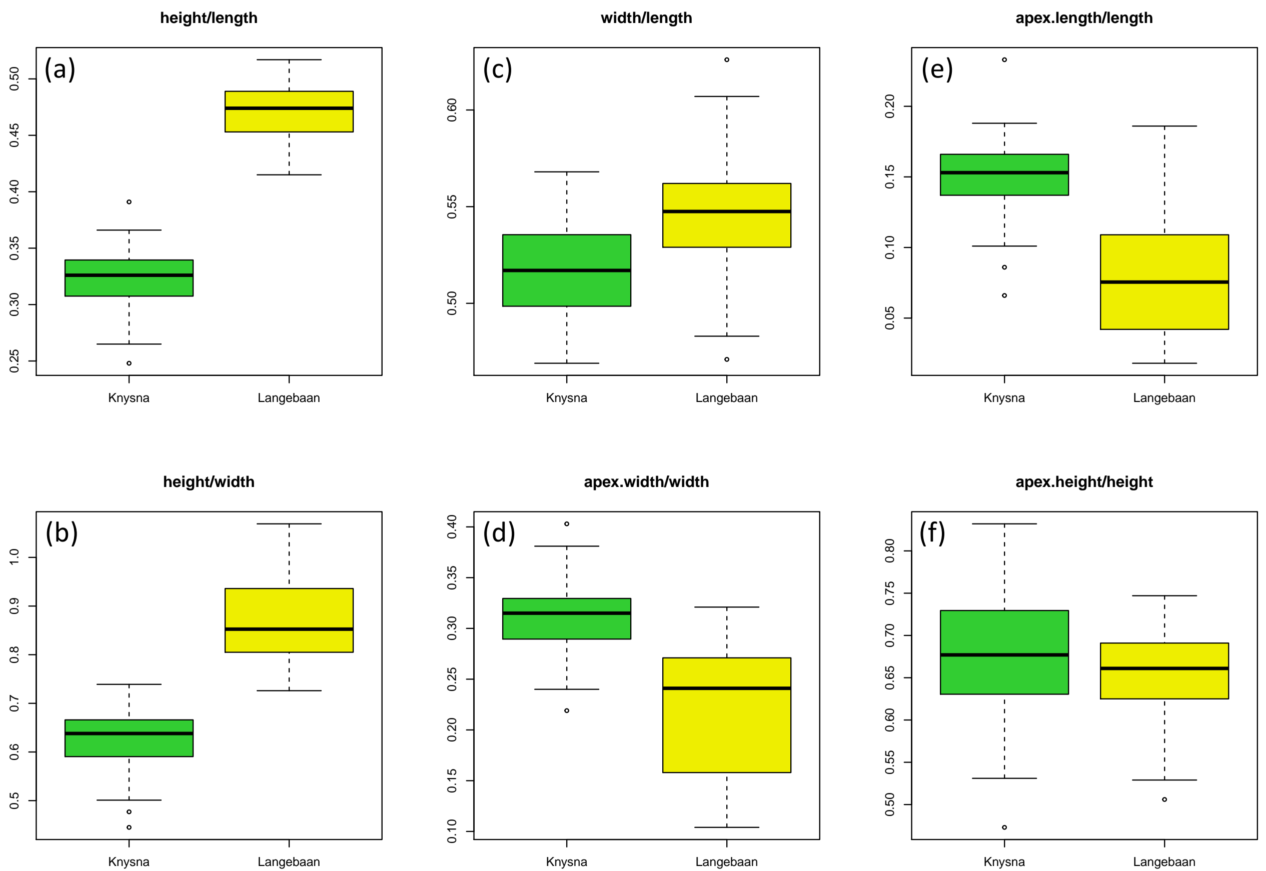

Figure 3.8 Boxplots for the morphometric ratio data for the two lagoons. Shell height/shell length (a), shell height/shell width (b), shell width/shell length (c), apex width/shell width (d), apex length/shell length (e) and apex height/shell height (f).

Shell mass was positively related to length in a logarithmic (log10) relationship for both populations (R^2 Langebaan = 0.91; R^2 Knysna = 0.84) (Table 3.3). All other regressions among mass, length, width and height variables for each of the populations were positive and significant, with R^2 values spanning 0.63–0.95 (Table 3.3).

Log-log regression results for mass/length indicate that the slopes of the regression lines were not significantly different between the two populations (p = 0.243). However, the difference between the intercepts of the two regression lines (Langebaan: -1.369; Knysna: -1.875) was highly significant, reflecting the fact that the mass of shells of any given length was significantly greater at Langebaan than Knysna (Figure 3.9).

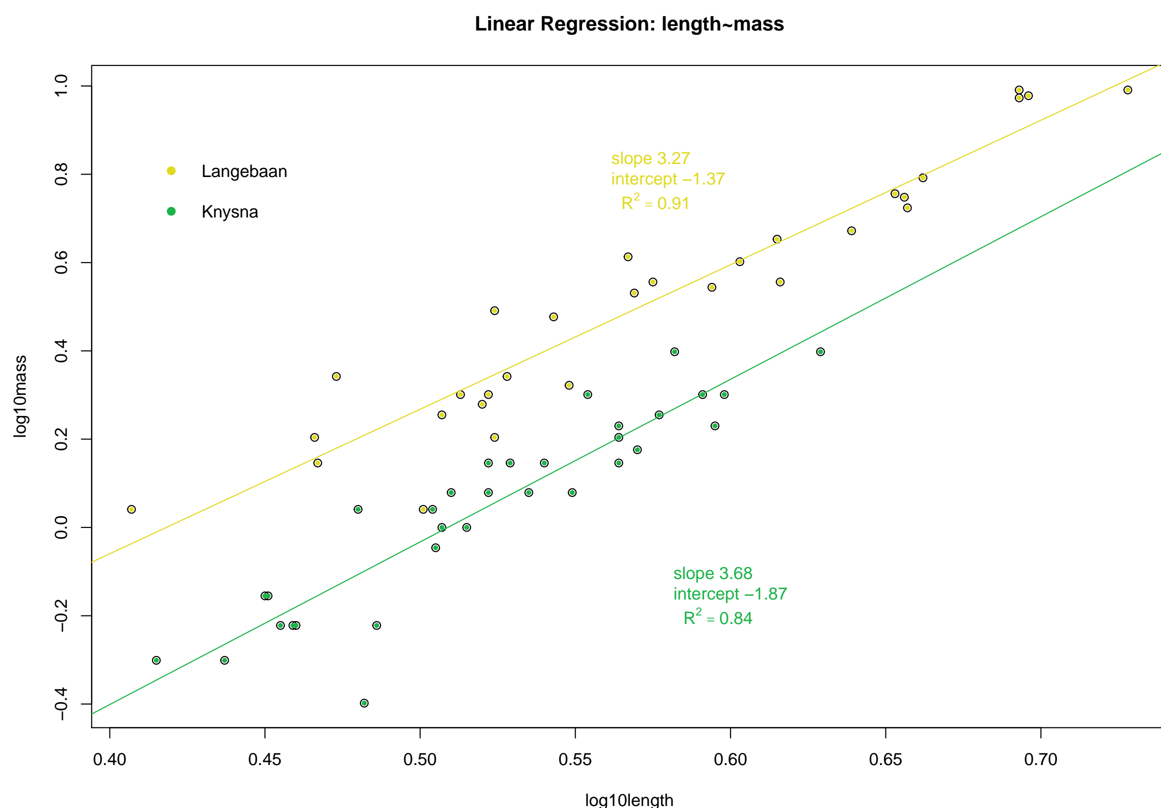

Figure 3.9 Linear regression plots with slopes, intercepts and R^2 values for the variables length and mass for the two populations.

(B) Multivariate statistical analysis

The Canonical Discriminant Analysis established that in multivariate space the two populations are morphologically well separated with the main multivariate differences being relative height and width of shells. The single Canonical Variate possible for two groups has a high and significant Canonical Correlation (0.95; Wilks' lambda = 0.104, ($P<0.0001$; Pillai's Trace = 0.896, $P < 0.0001$; Hotelling-Lawley = 8.601, $P< 0.0001$; Roy's Largest Root = 8.601, $P<0.0001$) (Table 3.4 a, d) with group membership, and scores of

individuals from the two sampling areas show no overlap on this Variate. In Cross-validation analyses (Table 3.4c) no individuals were assigned to the incorrect group.

Table 3.4a-d Summary of Canonical Discriminant Analysis for the two sites. See text for explanation.

a)

Eigenvalue	Percent	Cum Percent	Canonical Corr
8.60115386	100.0000	100.0000	0.94649134

b)

Scoring Coefficient	Log Length	Log Height	Log Apex Length	Log Width	Log Apex Width	Log Apex Height	Log Mass
Canon1	-56.90946	27.085635	0.6873605	27.049786	-3.609173	-2.338655	2.4056161

c)

Counts: Actual Rows by Predicted Columns	K	L
K	32	0
L	0	30

d)

Test	Value	Exact F	NumDF	DenDF	Prob>F
Wilks' Lambda	0.1041541	66.3518	7	54	<0.0001*
Pillai's Trace	0.8958459	66.3518	7	54	<0.0001*
Hotelling-Lawley	8.6011539	66.3518	7	54	<0.0001*
Roy's Max Root	8.6011539	66.3518	7	54	<0.0001*

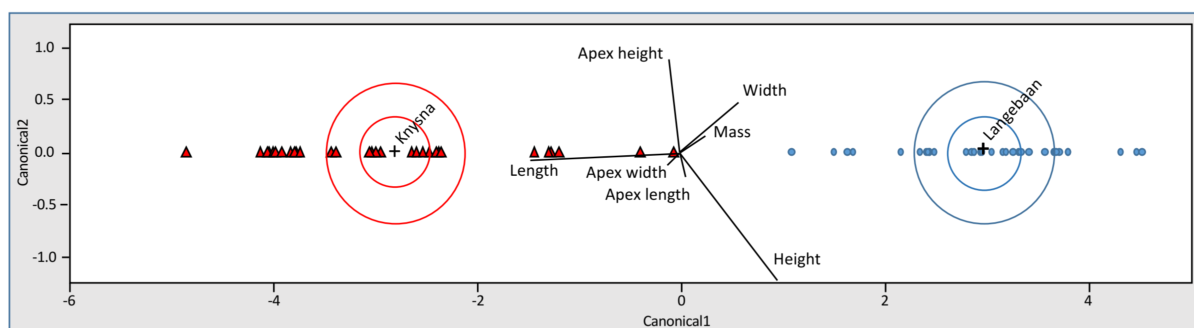

Figure 3.10 Canonical plot for linear discriminant analysis showing the canonical correlation structure of the variables (covariates) for the morphometric shell data. The observation points are red for Knysna (n = 32) and blue for Langebaan (n = 30). The plus marker denotes each multivariate mean. The 95% confidence levels (larger circles) do not intersect, indicating significant differences between the two lagoons. The 50% contour (smaller circles) contains roughly 50% of the observations. From the positions of the rays that represent the covariates, Log_{10} Height (mm), Log_{10} Width (mm), Log_{10} Mass (mg) and Log_{10} Apex Length (mm) are all positively associated with Canonical1.

The biplot axes (Figure 3.10) show two canonical variables, note however, there were only two categories in my data and thus only one canonical variable.

Log values of Height, Width, Mass and Apex Length were all strongly positively associated with Canonical Variate1, whilst Length and Apex Width and Height show high negative loading on the variate (Table 3.4b). This reflects the contrasts noted above in the relative shell proportions.

Figure 3.11 (Langebaan) and Figure 3. 12 (Knysna) show dorsal and lateral views of some of the individuals examined in this chapter. Key features that emerged distinguishing Langebaan from Knysna specimens were: (1) larger size at Langebaan; (2) heavier shells relative to shell length; (3) smaller apex-length to shell-length ratios at Langebaan, so the apex was closer to the back of the shell (and even 'overhung' the back in some instances; (4) a more excentric position of the apex at Langebaan, so the apex was more lateral and the apex-width to shell-width ratio was smaller; (5) relative shell height was less at Knysna, as reflected in a low ratios of shell-height to shell-width, and shell-height to shell-length. Apart from these quantifiable variables, Knysna shells were a uniform lustrous brown, while Langebaan specimens were duller and had radiating pale striations.

Figure 3.11 Dorsal and lateral views of *S. compressa* shells from Langebaan.

Figure 3.12 Dorsal and lateral views of *Siphonaria* shells from Knysna.

Specimens of Knysna individuals were selected as intended holotype (Figure 3.13) and intended paratype specimens (Figure 3.14), and will be deposited in the KwaZulu-Natal Museum, Pietermaritzburg, South Africa.

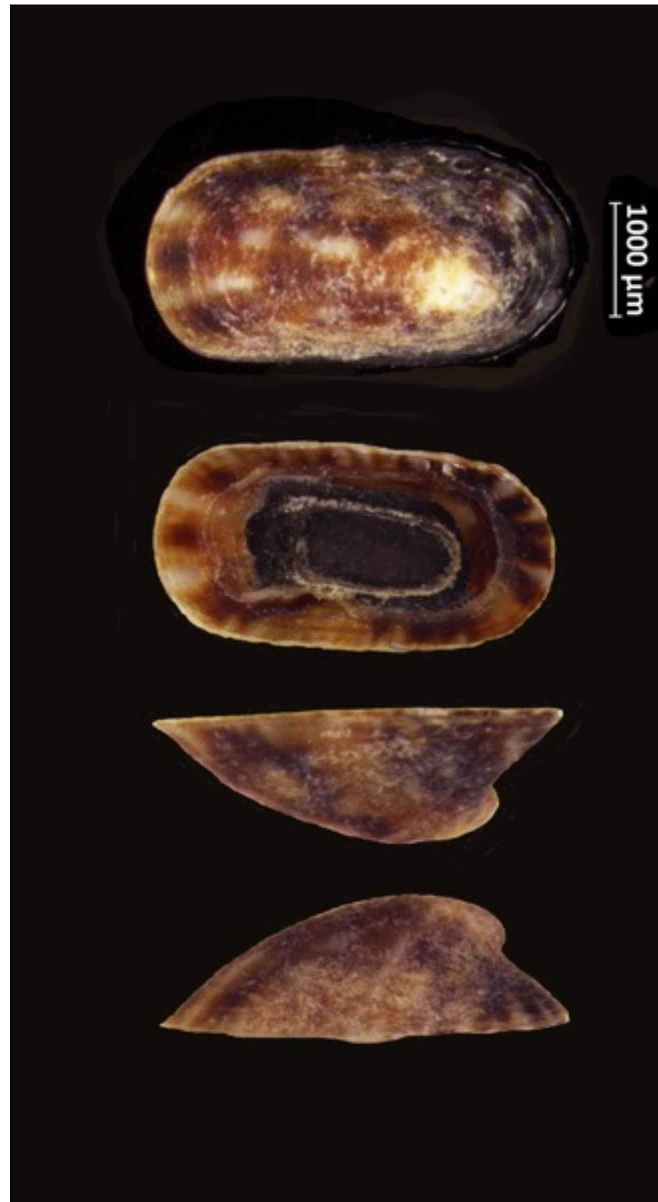

Figure 3.13 Dorsal, ventral, left and right photographs of Knysna *Siphonaria* nov. sp. 3 holotype.

Figure 3.14 Dorsal, lateral and ventral photographs of Knysna *Siphonaria* nov. sp. 3 paratypes.

Comparison of the two species
On the basis of both genetic and morphological shell differences I consider the material from Knysna to be a new species, previously assumed to be conspecific with *Siphonaria compressa*, found in Langebaan. The two species can be separated genetically by the absence of any shared haplotypes, and are also distinct from all other *Siphonaria* species I examined (see Chapter 4). They are also morphologically recognisable as separate species on the basis of shell shape, size and weight. Their shells can be distinguished most readily by the ratio of shell height/shell length (SH/SL) (Figure 3.8a), which spans 0.415-0.517 (median 0.475) for Langebaan and 0.248-0.391 (median 0.325) for Knysna, with no overlap. The ratio of shell height/shell width (SH/SW) (Figure 3.8b) is also distinctive, although with a marginal overlap, spanning 0.726-1.069 (median 0.850) for Langebaan and 0.445-0.739 (median 0.640) for Knysna. On present evidence, geographic location is the simplest means of identification, *Siphonaria* nov. sp. 3 being confined to Knysna with a possibility of occurrence in Keurbooms Estuary, and *Siphonaria compressa* limited to Langebaan Lagoon.

Etymology: The intension is to name the Knysna species after Dr Brian Allanson who discovered and identified both the species living in Langebaan (in 1959) and in Knysna (in 2006), but formal naming and description of the species will follow in a publication pursuant to this thesis.

Description: Compared to *S. compressa* from Langebaan, the Knysna shells have a lower height relative to both length and width; the apex of Knysna specimens lies nearer the midline, and also further forwards than in Langebaan individuals; overall size at Knysna is also smaller than at Langebaan and the shell less robust and lighter. Knysna shells were a uniform lustrous brown, almost transparent, horn-like and corneous with a more delicate, finer sculpture, while Langebaan specimens were calcareous, duller and had radiating pale striations. No siphonal groove is present in the shells of either species.

Dimensions: Largest specimen examined: shell mass 2.5 mg, shell length 4.3 mm, shell width 2.1 mm, shell height 1.4 mm.

Distribution and habitat: Known only from Knysna Lagoon, South Africa. Material examined: 44 specimens collected from Leisure Isle -34.0608, 23.0515; Bollard Bay -34.0639, 23.0665.

3.4 DISCUSSION

The data provide clear genetic and morphological evidence that the populations of what is currently regarded as *S. compressa* at Knysna and Langebaan represent distinct species, between which there is long-standing absence of genetic exchange. I recognize that size alone is not a reliable means of distinguishing species, because environmental conditions can influence size, with lower temperatures and higher productivity being associated with larger sizes (Teske *et al*. 2009); but the differences in proportions, shapes and colours of these sister species remain distinctive.

Here I discuss the diversity, age, size, possible mechanisms of separation and morphometric differentiation between the two populations. Finally, I will address the conservation management implications of the findings, focusing on three points: (1) an evaluation of the vulnerability of the two populations, (2) consideration of the two populations as genetically distinct separate species and (3) the effects thereof on the practicality of various conservation measures.

Population distinction and diversity

The primary question that this chapter addresses is whether these populations are separate species or not. The molecular data show that the two populations have no shared haplotypes which suggests they are completely genetically isolated, with the isolation event occurring around 730 000 years ago, as estimated from my mitochondrial gene analysis. Next-generation analyses are underway to confirm this, but on present evidence, the Langebaan population is older and has more molecular diversity than does that of Knysna. A number of lines of evidence all point towards recently expanding populations at both sites, namely the 'starlike' nature of the haplotype genealogy, smooth unimodal mismatch distributions, negative Tajima's Ds and low R2 values. Negative Tajima's Ds are associated with a population expansion after a bottleneck or a recent selective sweep. The latter is, however, unlikely, as mitochondrial base changes, especially in CO1, are constrained and are thus mostly synonymous (James *et al*. 2016). We also know *a priori* that the populations have repeatedly undergone regular population crashes (Angel *et* al. 2006, B. Allanson pers. com.), and it is likely that they have been subjected to genetic bottlenecks.

Uni-, bi- and multivariate analyses confirm there are clear morphometric differences between the two populations. As they occupy identical habitats, eelgrass in the intertidal zone, the differences between the two geographical areas that have emerged are unlikely to be phenotypic responses to habitat differences.

Population age

The split between the two populations is estimated to have occurred around 730 000 years ago with an HPD interval of 300 000 to 1.3 million years ago (mya) (Table 3.2).

This falls in the Late Pleistocene or Chibanian stage (0.126 - 0.781mya) when sea level fluctuations were at their greatest, spanning roughly from -150 m to +18 m relative to present sea level (Spratt & Lisiecki 2016). As sea-level changes lasting 2000 years or less would not be captured due to signal-to-noise ratios in the sea-level reconstruction techniques (Kopp *et al.* 2013), this implies that even greater changes could have occurred. At around 2.5 mya, the transition between the Pliocene and the Pleistocene epochs, there was a change in the behaviour of the global climate and a general trend downward of sea level from about 3 mya to the present. In addition, in the terminal Pleistocene there was substantially greater variation and fluctuation of sea levels from 600 thousand years ago (kya) to the Last Glacial Maximum (Figure 3.15). This time frame corresponds with my estimated IMa2 dates, with lowstands or sea level minima at that time well positioned to be the reason for the split between the two populations: the sea level would have been exceptionally low and the coast line and estuarine environments would have been dramatically altered by changes in both flow rates and water chemistry. These would have resulted in habitat loss, population fragmentation and the development of dispersal barriers.

In fact, the four major lowstands corresponding to glacial maxima where sea levels dropped to over 100m below modern sea levels, could have singly or together provided the environmental conditions conducive to initiating isolation and speciation, and subsequently maintaining the required physical barriers for allopatric speciation.

Even given the wide range of estimates, it is clear that divergence between the two populations is ancient. This allows dismissal of the possibility that either of the populations arose recently by introduction of specimens by human-related events such as transport by boats or via aquaculture (Haupt *et al.* 2010, Peters *et al*. 2019)

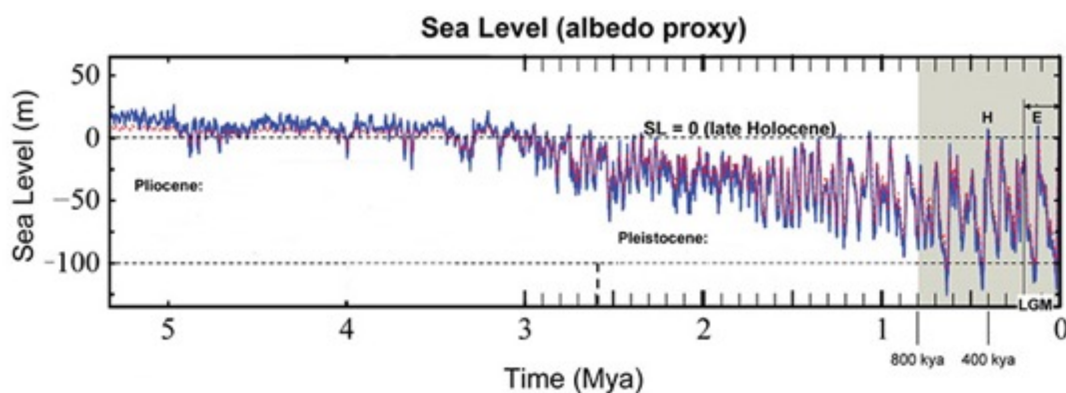

Figure 3.15 Sea level change during the last 5 million years. Sea level was estimated by means of albedo proxy, a method of determining past ice cover and sea level of the planet by means of the percentage reflection of solar radiation. Published on Coastal Processes, Hazards, and Society (https://www.e-education.psu.edu/earth107) by Dr. James Hansen at Columbia University's Earth Institute. Copyright Root Routledge, but available for non-commercial distribution.

Population size

The contemporary population sizes I estimated were 1.3 million for Langebaan and 1 million for Knysna with ancestral populations of about 340 000 individuals at both sites (Table 3.2). The estimate of a small ancestral population of 340 000 individuals does not seem to support the hypothesis of a larger panmictic population. However, the confidence interval is zero to 46 million individuals which makes it difficult to interrogate this interpretation further. The large confidence intervals for contemporary size (680 thousand to 2.4 million for Langebaan and 530 thousand to 2 million for Knysna) estimated from the IMa2 analysis are most likely due to the use of effectively only one locus and unavoidable inaccuracy in the chosen mutation rate. The relatively small sample size is also a factor that can lead to difficulties in interpretation.
In addition, the estimates for the most recent common ancestor (Langebaan, 300,000 years ago and Knysna, 150,000 years ago) and the predicted extant population sizes of 3 million for Langebaan and 5 million for Knysna established from the Bayesian skyline plots, are dependent on the number of haplotypes sampled and the current population size and, as such, can fluctuate. Indeed, Grant (2015) cautions against over-interpretation of both mismatch distribution and Bayesian skyline plot analysis as both methods are error prone and thus problematic for inferring historical demography.

Given the wide confidence intervals, it is nevertheless possible to speculate that effective population sizes were previously much larger than they are at present and could possibly have extended over more than the two estuaries. This would suggest a larger, more widespread ancestral coastal population present prior to the increased sea level volatility which caused portions of coastline to become isolated over time, subsequently leading to extinction in the intervening estuaries and allowing the evolution of two separate sister taxa. My thorough search of the neighbouring Keurbooms Estuary in March 2008, the only other site where shells had been reported (Herbert 1999), was in the hope of finding evidence of this in the form of another remnant pocket of individuals; but none were found, and it is likely that the population evidenced there by drift shells is locally extinct.

The Knysna seahorse *Hippocampus capensis* is another species that is largely confined to Knysna. It, too, has been analysed genetically and shows a pattern completely different from that of *S. compressa*, with a close genetic relationship between the Knysna population and two other populations that appear to have recently established in other (nearby in this case) estuaries (Mkare *et al.* 2021).

Population origins

In an exercise in which estuaries were grouped on the basis of bird community structure, Knysna and Keurbooms estuaries both fell in the same category (large, open, with appreciable mudflat areas with a high wader count) (Turpie & Clark, 2007). Although Langebaan was not addressed in this particular report, it is the most important wetland for waders in South Africa (BirdLife South Africa 2016), suggesting

that these characteristics may be considerations in hypothesising estuaries in which *S. compressa* may have thrived. In view of the intolerance of *S. compressa* to variations in salinity (Wilson *et al.* 2009), a critical feature is the need for relatively constant seawater salinities, which tends to be the case in tidally dominated lagoons such as Langebaan and the lower basin of Knysna.

The two populations may have diverged following separation, and adapted to local circumstances. Given the overlapping ranges and variability of salinities in the two systems, it is unlikely that adaptation to salinity would have driven this. However, the two populations do lie in different ecoregions with different thermal characteristics: Langebaan falls in the Cool Temperate Namaqua Ecoregion, and Knysna in the Warm Temperate Agulhas Ecoregion (Sink *et al*., 2012, Branch & Branch 2018). Teske *et al.* (2019) have previously suggested that thermal selection may drive ecological speciation. Emami-Khoyi *et al.* (2021) have demonstrated that genetically unique ecotypes exist within different species that occur in these two bioregions. For one of these, namely the mudprawn, *Upogebia africana*, these genetic differences are adaptively associated with different thermal tolerances; and thermal stress may exclude ecotypes in each bioregion from areas beyond their tolerance. They regard this as a case of incipient speciation; and the differentiation between *S. compressa* and *Siphonaria* nov. sp. 3 may represent the end point of such thermally-mediated divergence, with speciation now complete.

A possible alternative theory for the origin of two taxa would be that one population colonised the other, possibly by the long-distance rafting of individuals on severed eelgrass blades. Rafting has been suggested for the dispersal of other species, even over distances as great as between Patagonia and New Zealand, as proposed for the slug *Onchidella marginata* (Cumming *et al.*, 2014). From experimental work on the Knysna population it was established the spawn is only deposited on eelgrass and hatches into completely metamorphosed juveniles after 13 (at 25˚C) to 18 days (at 17˚C). There is a high percentage hatch rate and the eggs and young seem relatively robust under various laboratory conditions (Allanson & Msizi 2010). Thus, it is biologically possible for rafting to have occurred. However, the higher molecular diversity present in the Langebaan population would suggest that the direction of any colonization was from Langebaan to Knysna. This raises the question of how this would be achieved given the prevailing direction of the Agulhas current (established 5 mya), the movement of Agulhas rings and the Benguela upwelling, which all flow in the opposite direction (Lutjeharms 2006). Research from deep-sea cores suggests, however, that in the Late Pleistocene the subtropical convergence was north of Durban and not in its current southerly position. This would mean that subantarctic water flowing eastwards would have been closer to the continent (Bé & Duplessy 1976). Other work suggests that during the winter the Agulhas was modified and may have been replaced by water from the south with possible wind reversal affecting surface circulation (Hutson 1980).

Another possibility is that birds may have served as a vector (Green & Figuerola 2005; Green *et al*. 2007) for transfer between Langebaan and Knysna. Birds are,

however, unlikely to be a 'standard vector' (*sensu* Nathan *et al*. 2008) as neither *S. compressa* nor its host *Zostera capensis* (to which the limpet may remain attached) have any adaptations to facilitate attachment to birds. Transport through the guts of birds seems even less likely, given the fragility of the limpets.

The species concept

A further consideration is the matter of the species concept adopted. Both genetic divergence and reproductive isolation are necessary for the strict biological species definition. As three million years of allopatric speciation of marine shrimp, fish and sea urchins has been shown to be insufficient for the emergence of full reproductive isolation (Lessios 2008), it is possible that the two limpet populations still may be able to interbreed if they are indeed remnants of one larger panmictic population that was severely reduced – or even two populations related by an initial long-distance colonisation event. The reproductive isolation question could be further explored by mating experiments. Given the ease with which laboratory work was performed on the Knysna population (Allanson & Msizi 2010), this would be feasible. However, as I outline in Chapter 1, I would suggest using a broader, more lenient approach to species, as applied in the unified species concept, which focuses on species less as fixed taxonomic entities and more as constantly evolving lineages (De Queiroz 2007). Indeed, in a recent review, Fišer *et al.* (2018) call for a paradigm shift in the species concept to allow cryptic species – which have been shown by molecular delimitation methods to be both common and widespread – to be incorporated into biodiversity science.

Even with this more relaxed approach to speciation, the potential for interbreeding and the possibility of panmixis seem unlikely. With no shared haplotypes – making them quite distinct molecularly from each other – and no migration (and thus no gene flow) between these two populations, and with the divergence time between them so substantial, it seems more likely that they are distinct sister taxa representing different species and in all likelihood unable to interbreed.

Morphometric differences

There are clear morphometric differences between the two populations as shown by uni-, bi- and multivariate analyses. The populations can be most easily separated on the basis of the following ratio: shell height/shell length (SH/SL), which is the most diagnostic of the ratios, followed by shell height/shell width (SH/SW). The Knysna shells are shallower relative to both length and width, compared to those from Langebaan; the apex of Knysna specimens lies nearer the midline, and also further forwards than in Langebaan individuals; and most distinctively, the overall size at Knysna is smaller than at Langebaan and the shell less robust. Differences between individuals from the two different geographical areas are thus distinct and diagnostic. This reinforces the conclusion that they are distinct but cryptic species. Allanson and Herbert (2005) could find no differences between the genitalia of the two; but did note

that the radula formula for Knysna speciments spanned 9:1:9 to 12:1:12, less than the 15:1:15 recorded for the type specimen from Langebaan.

In 2018, additional searches were made in Knysna to test whether the distinct differences in size between Langebaan and Knysna individuals evident in the initial samples upon which the morphometrics analyses were based were simply because the samples from Knysna were taken after floods had depleted the population, leaving a 'young' population with only small individuals. One hundred additional animals were measured in Knysna from Bollard Bay and Thesen's Island, the original sampling sites. There were no large individuals and sizes at both sites in Knysna remained consistently smaller than at Langebaan.

It is, of course, possible that both sets of data for Knysna (2008 and 2018) were for 'young' populations, but in view of the fact that the limpet was abundant in 2018, that is unlikely for that year at least. Densities were very high: in 10x10 cm areas, counts were 86, 42, 65, 42, 37 at Bollard's Bay. Numbers at Thesen's Island were lower but still quite impressive: 21, 15, 22, 25, 10 (unpublished personal data). This is in contrast to the numbers in 2008 when the original samples were collected: individuals were scarce, thinly spread, very patchy and extremely difficult to find.

It has been experimentally established, for the Knysna population at least, that they rapidly reach maturity, and are high-frequency spawners with a very short period between spawning and recruitment. In fact, the short time for reproductive length to be reached (between 120 to 170 days), together with the shortest time of development (for South African siphonariid direct developers) may indicate a selective advantage that enables rapid regeneration in an unstable environment (Allanson & Msizi 2010, Allanson & Fearon 2012). It seems that a feature of the mollusc in both Knysna and in Langebaan (Angel *et al.* 2006) is the ability to recover rapidly from very low densities.

Given that it appears that the two populations are in fact two different species it would be of interest to repeat the Knysna spawning experiments with Langebaan individuals to investigate if its development there is similar. This may explain the fact that Langebaan shells are significantly heavier than those from Knysna despite sharing a similar habitat. Smaller, lighter shells may be due to recovery following periodic flood events depleting the Knysna population, but would not explain the greater weight or relative height for Langebaan specimens of a given shell length.

Conservation and management implications

I turn now to the conservation management implications of the findings. Both climate change and increased human activity causing habitat reduction are having negative effects on marine ecosystems globally (Doney *et al.* 2012). Separate evaluations of the vulnerabilities of the two populations are necessary as each population is under threat for different reasons.

Although Langebaan has RAMSAR status (Marx & Liebenberg 2019) it faces anthropogenic threats related to (1) habitat destruction from human foot traffic from expanding tourism, (2) microalgal fouling of the eelgrass, the limpet's symbiotic host

plant; (3) pollution and iron-ore deposition at the nearby ore jetty at Saldanha (Beckley 1981); (4) alien species (Robinson & Griffiths 2002; Sadchatheeswaran *et al.* 2015); and (5) dredging associated with harbour construction that has increased siltation in the lagoon (Kruger *et al.* 2005). The eelgrass beds are exposed at low tide and any increase in shore-based tourist activities will subject them to greater disturbance, especially in the summer holiday period. *Siphonaria compressa* is restricted to the surfaces of the eelgrass and, given the weak wave action and gentle tidal movement in the shallow edges of the lagoon where the eelgrass beds occur, the conditions are conducive to both trampling and sedimentation on the blades. Harmful algal blooms caused by the picoplankton *Aureococcus anaphagefferens* carried by ballast water, first recorded in Langebaan in 1998, have caused shading of the eelgrass in the past (Stephen & Hockey 2007). A further feature is the mollusc's restriction to an extremely narrow habitat range on the lower edges of *Zostera* beds to which it is constrained by burrowing activities of the sandprawn *Kraussillichirus kraussi* at the bottom, and physical stressors at the top (Angel *et al.* 2006).

The eelgrass in Langebaan has fluctuated over the last 34 years of monitoring, such that twice the *S. compressa* population has been in danger of extinction: once during harbour construction at Saldanha when silt smothered the eelgrass beds, and once for an unknown reason (Angel *et al.* 2006). In addition to fluctuating abundance and reflecting a global trend, the cover of *Zostera* has declined by 38% over the last 50 years at Langebaan (Pillay *et al.* 2010).

Even though Knysna is the highest-ranked South African estuary with regard to conservation importance (Turpie *et al.* 2002), the Knysna population is similarly threatened by tourism, trampling during bait harvesting and by other anthropogenic activities in its catchment (Claassens 2016). It is additionally vulnerable to periodic flooding of the Knysna River (Allanson & Msizi 2010). Tourist foot traffic and recreational water activities cause both physical disturbance and degradation of the eelgrass beds, and suspend additional particulate matter in the relatively shallow water, which settles on the surfaces of the eelgrass, hampering both attachment and grazing by the limpet. Smothering by large macroalgal blooms or 'green tide' experienced in Knysna Lagoon since 2014 are another recent concern (Allanson *et al.* 2016). Severe floods detach entire sections of upstream riverbanks and scour whole eelgrass beds from the lagoon and wash them out to sea (personal observation). But the biggest threat is likely to be periodic reductions of salinity associated with floods. Given the severity of the relatively recent flood events at Knysna it is remarkable that *S. compressa* is once again thriving there in mid-2018 (see data on densities mentioned above).

The most striking environmental differences between the two populations is that they are found in different marine biogeographical provinces. Langebaan is in the cool-temperate Namaqua province while Knysna is in the warm-temperate Agulhas province (Sink *et al.* 2005). For this reason, they might have different thermal tolerance ranges. Both populations would, however, be subjected to similar upper temperatures when the *Zostera* beds are exposed at low tide. It has been established that Knysna larvae are capable of hatching at 17°C (Allanson & Msizi 2010). No thermal physiological

experimental work has been done on Langebaan individuals. Once again, this is a relatively simple laboratory experiment that could be performed.

A second difference is that Langebaan has no freshwater input and thus has a relatively constant environment, while Knysna is subject to riverine inflow and periodic flooding, a repeated near-extinction pressure. Tests on the Langebaan population of *S. compressa* show it is unique in the genus in that it has a narrow salinity tolerance range (Wilson *et al.* 2009). As this narrow tolerance is partly related to the small size of the individuals, it is likely that this trait will be shared by the Knysna population. If this is the case, then the population will be especially vulnerable due to the increasingly common flooding events in Knysna, which are a downstream result of climate change. For example, after the December 2004 floods, densities fell from a patchily abundant level of 358 m^{-2} when they were first discovered, to 32 m^{-2} (Allanson & Herbert 2005). The fact that the Knysna population was first discovered only recently, implies long periods of vanishingly small numbers that remained undetected in spite of estuarine research having been undertaken in the estuary for decades.

This brings me to the evaluation of the viability of various conservation measures that might be applied to the two populations in the event of a catastrophic decline in numbers or even extinction at either site. Given that there have been rapid population declines and rises recorded in recent years at both sites (Angel *et al.* 2006, Allanson & Msizi 2010), this is a strong possibility. Such population collapses are a concern as genetic bottlenecks reduce genetic diversity (Luikart *et al.* 2002), and inbreeding reduces resistance to disease and increases extinction, especially in stressed populations (Frankham 2005). Demographic rescue (adding individuals to increase a population's size) and genetic rescue (adding variation to reduce inbreeding and ensure continuous adaptation) (Hufbauer 2015) were, together, both ultimately successful in reducing extinction risk in laboratory experiments with flour beetle (*Tribolium castaneum*); and recent meta-analyses indicate that benefits of genetic rescue can persist until the F3 generation (Frankham 2016).

Prior to my research, various supportive conservation measures such as these could have been considered in the event of catastrophic extinction scenarios, given the assumption that the two populations were of the same species. However, all the evidence presented here strongly suggests these two populations are genetically and morphometrically distinct evolutionary lineages that do not share any haplotypes and, as such, would need to be dealt with as distinct Evolutionarily Significant Units (ESUs) that are likely adapted to different environmental conditions and represent cryptic species. This has been shown for numerous other invertebrates in the region (Teske *et al.* 2006, 2008, Zardi *et al.* 2011, von der Heyden *et* al. 2011, Papadopoulos & Teske 2014). Thus, it would be neither practical nor desirable to use augmentation measures such as inter-regional translocations, which in other cases have been used to increase genetic diversity and reduce inbreeding in isolated populations with a high risk of extinction. Genetic rescue or outcrossing to reverse inbreeding (Frankham 2016) and maintain fitness such as fecundity and survival is a restocking intervention suitable for genetically indistinguishable populations; but it is clear from my research that the

Langebaan and Knysna populations are genetically distinct species and, as such, they will need to be managed separately. The only caveat here is that the seemingly extinct Keurbooms population may have been genetically the same as Knysna given its proximity and, if so, then translocation would be feasible. Interbreeding between individuals from the two populations with substantial genomic differences - even if it is possible - could have negative consequences for fitness, if not in the F1, then in the F2 generation (Edmands 1999).

However, new research has found evidence that outbreeding is less of a hindrance than inbreeding; identifying the inbreeding coefficient would allow a firm decision to be taken on whether augmentation is desirable (Ralls *et al.* 2018). A further factor to consider here is that all *Siphonaria* species are hermaphroditic; and hermaphroditic species are known to exhibit less inbreeding depression than obligatory outcrossing species (Dolgin *et al*. 2006, Charlesworth & Willis 2009). A recent laboratory study with small populations of inbred Trinidadian guppies (*Poecilia reticulata*) found that in augmented populations both adult recruitment and total abundance increased with adaptively similar but genetically divergent supplementation (similar to the *S. compressa* scenario), yielding a larger increase in abundance than in populations augmented with adaptively divergent but genetically similar individuals (Kronenberger *et al.* 2017). This evidence suggests that genetic rescue potentially can be successful even if the populations are highly divergent, and motivates for experimental work to be initiated on the two populations.

Given the observed population fluctuations at both sites and the evidently extremely low numbers experienced periodically, one would expect the repeated population bottlenecks and founder effects would have severely reduced genetic variation, with inbreeding depression having an effect on the viability of the species. However, Chambers *et al.* (1998), found within-population variability for *S. compressa* from Langebaan to be the highest out of the 12 siphonariids they studied with RAPD fingerprinting. In addition, my data for both populations combined yielded a nucleotide diversity (0.0103, n=83) similar to more common and widespread South African species like *S. serrata* (0.0141, n=18, 18 sites) and *S. capensis* (0.0201, n=245, 20 sites) (see Chapter 2). It is possible this higher diversity is a key factor contributing to the resilience and survival of the two populations in the face of repeated near-extinction fluctuation.

An alternative method of conservation could include the creation of artificial habitat. This technique was successfully used to create additional new habitat for the endangered Knysna seahorse (*Hippocampus capensis*) endemic to the Knysna, Swartvlei and Keurbooms estuaries (Claassens 2016). 'Mixed vegetation on sediment' (which included *Zostera capensis*) was one type of artificial substratum monitored during the experiment.

Angel *et al.* (2006) showed that if the bioturbator *Kraussillichirus kraussi* is experimentally excluded from plots of translocated *Zostera capensis* in Langebaan, the eelgrass grows rapidly, and subsequent 20-fold increases in *S. compressa* densities were recorded in these plots. This suggests that artificial creation of eelgrass habitat

could be a promising methodology to enhance populations of eelgrass-dependent *Siphonaria* species.

This situation of two endangered and rare molluscan sister species in two lagoons in one country is unique and we have an obligation to ensure that both areas are carefully and thoughtfully managed and protected. Recognition that the two populations are separate species with separate conservation needs should inform decisions regarding the management of the different ESUs, particularly given the hotspot traits of high endemism and high environmental threat common to both lagoons. Ex situ measures are, however, likely to be difficult in view of the tight association of both species with *Zostera capensis*.

Conclusions

The hermaphrodite siphonariid mollusc, *Siphonaria* nov. sp. 3 is described from Knysna Lagoon, on the southern coast of the Western Cape, South Africa, and is distinguished from its close sister species *S. compressa* on the basis of both genetic and morphological shell differences.

There are therefore two small members of the genus found in South Africa. *Siphonaria* nov. sp. from Knysna has similar habits to its sister taxon *S. compressa* Allanson 1958, which is restricted to Langebaan Lagoon on the west coast of South Africa. Both species habitually attach to the seagrass *Zostera capensis* growing in tidal flats in each lagoon. This is distinctive for these two species in South Africa.

This chapter set out to determine if there are genetic and major shell differences between the populations in Langebaan and Knysna lagoons, and confirmed that this is the case. Potential differences in radular morphology, internal anatomy, sperm morphology and shell microsculpture are not addressed here, but may aid in further distinguishing the species.

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Appendix 3.1

Raw morphometrics data. "L" refers to Langebaan samples and "K" refers to Knysna samples. Measurements are in millimeters (mm), weight in milligrams (mg). Shell length (SL, greatest distance between anterior and posterior end), shell height (SH, greatest vertical distance from the top of the shell to the plane of the aperture), shell width (SW, greatest distance perpendicular to the anterior-posterior axis), apex length (AL, distance from apex to posterior shell margin, apex width (AW, distance from apex to maximum shell width) and apex height (AH, distance from to apex to plane of the aperture).

sample	length mm	height mm	apex length mm	width mm	apex width mm	apex height mm	mass mg
L1	4.964	2.460	0.544	2.485	0.375	1.767	9.5
L2	5.350	2.563	0.388	2.642	0.274	1.641	9.8
L3	4.354	2.102	0.330	2.352	0.524	1.437	4.7
L4	4.117	2.074	0.398	2.194	0.553	1.424	4.5
L5	3.762	1.816	0.137	1.816	0.217	1.172	3.6
L6	4.5	2.214	0.45	2.45	0.605	1.654	5.7
L7	4.538	2.02	0.409	2.293	0.308	1.491	5.3
L8	4.93	2.38	0.26	2.465	0.452	1.462	9.8
L9	4.135	1.962	0.337	2.303	0.317	1.298	3.6
L10	4.525	2.073	0.311	2.481	0.626	1.369	5.6
L11	4.937	2.485	0.291	2.325	0.325	1.553	9.4
L12	2.974	1.401	0.117	1.861	0.476	0.825	2.2
L13	3.492	1.709	0.65	1.848	0.443	0.864	3
L14	3.689	1.835	0.068	2.095	0.307	1.17	4.1
L15	3.34	1.398	0.403	1.796	0.486	0.966	3.1
L16	3.704	1.665	0.277	2.068	0.563	1.141	3.4
L17	2.926	1.388	0.063	1.689	0.463	0.791	1.6
L18	4.596	2.375	0.136	2.369	0.374	1.411	6.2
L19	3.929	1.977	0.159	2.112	0.393	1.262	3.5
L20	4.007	1.928	0.122	2.19	0.529	1.271	4
L21	2.551	1.156	0.108	1.549	0.265	0.611	1.1
L22	3.168	1.314	0.442	1.811	0.581	0.908	1.1
L23	3.371	1.549	0.232	2.035	0.583	0.954	2.2
L24	3.327	1.487	0.363	1.845	0.467	1.027	2
L25	3.531	1.496	0.476	1.974	0.522	1.092	2.1
L26	3.339	1.443	0.459	1.824	0.509	0.987	1.6
L27	3.215	1.488	0.366	1.808	0.356	0.978	1.8
L28	2.931	1.327	0.271	1.648	0.47	0.881	1.4
L29	3.312	1.531	0.261	1.86	0.406	1.119	1.9
L30	3.256	1.57	0.146	1.841	0.552	1.039	2
K1	3.2	0.987	0.542	1.639	0.5	0.756	0.9
K2	3.062	0.993	0.493	1.477	0.457	0.71	0.6
K3	3.211	1.116	0.401	1.732	0.442	0.756	1
K4	3.431	1.193	0.527	1.73	0.576	0.891	1.2
K5	3.235	0.975	0.431	1.693	0.475	0.625	1.2
K6	3.035	0.876	0.51	1.647	0.515	0.552	0.4
K7	2.822	0.924	0.316	1.337	0.491	0.672	0.7
K8	2.819	0.989	0.397	1.417	0.571	0.643	0.7
K9	2.602	0.689	0.421	1.445	0.41	0.504	0.5
K10	3.714	1.307	0.604	1.811	0.589	0.818	1.5
K11	3.585	1.245	0.634	1.754	0.624	0.911	2
K12	3.541	1.152	0.573	1.808	0.513	0.727	1.2
K13	3.328	1.3	0.219	1.759	0.579	0.788	1.4
K14	3.02	1.106	0.705	1.715	0.565	0.792	1.1
K15	3.666	1.243	0.689	1.721	0.655	0.924	1.7
K16	2.848	0.918	0.449	1.473	0.545	0.563	0.6
K17	2.886	0.791	0.249	1.578	0.515	0.374	0.6
K18	3.191	0.947	0.477	1.758	0.642	0.628	1.1
K19	2.878	0.715	0.343	1.605	0.55	0.38	0.6
K20	3.277	0.935	0.496	1.745	0.569	0.597	1
K21	3.663	1.129	0.648	1.762	0.568	0.836	1.4
K22	3.776	1.23	0.652	1.873	0.449	0.915	1.8
K23	4.256	1.408	0.79	2.133	0.543	1.172	2.5
K24	3.383	1.147	0.483	1.874	0.574	0.789	1.4
K25	2.734	0.776	0.276	1.431	0.447	0.428	0.5
K26	3.965	1.238	0.51	2.06	0.452	0.856	2
K27	3.822	1.365	0.461	1.918	0.509	0.992	2.5
K28	3.464	1.25	0.516	1.778	0.494	0.822	1.4
K29	3.667	1.215	0.602	1.903	0.628	0.892	1.6
K30	3.896	1.198	0.56	1.874	0.566	0.792	2
K31	3.324	1.037	0.489	1.696	0.521	0.66	1.2
K32	3.938	1.319	0.665	1.942	0.612	0.949	1.7
K33	2.702	0.884	0.422	1.5	0.443	0.549	shell broke
K34	2.976	0.954	0.467	1.657	0.526	0.672	shell broke
K35	3.607	1.14	0.544	1.782	0.456	0.74	shell broke
K36	3.354	1.139	0.623	1.736	0.638	0.816	shell broke
K37	3.375	1.1	0.448	1.709	0.512	0.801	shell broke
K38	3.256	0.951	0.498	1.703	0.544	0.598	shell broke
K39	3.174	1	0.452	1.627	0.573	0.765	shell broke
K40	2.88	0.951	0.419	1.607	0.487	0.597	shell broke
K41	3.15	0.962	0.549	1.555	0.496	0.73	shell broke
K42	2.869	0.937	0.438	1.488	0.419	0.568	shell broke
K43	2.672	0.921	0.346	1.415	0.448	0.599	shell broke

Appendix 3.2

IMa2 estimates of divergence times and effective population size means estimated from multiple independent runs with the same parameters but different seeds for the two populations of extant *Siphonaria compressa*. Marginal distribution values are in demographic units. t0 = time of divergence between populations in years; q0 = number of females in Langebaan population; q1 = number of females in Knysna population; q2 = number of females in ancestral population; HiPt = ; HiSmth = the bin with the highest value after smoothing; Mean = ; HPD95Lo = 95% Lowest Posterior Density Interval; HPD95Hi = 95% Highest Posterior Density Interval. '?' indicates possible multiple peaks or a rough surface.

CO1

RUN1 seed 1234 gen 1952

Value	t0	q0	q1	q2
HiSmth	788813	1406963	1110160	313927
Mean	812803	1582648	1252383	10766185
HPD95Lo	309361 ?	801941	579338	0
HPD95Hi	1352740 ?	2480023	2011986	43807078

RUN2 seed 5678 gen 1952

Value	t0	q0	q1	q2
HiSmth	713470	1401256	1098744	371005
Mean	809433	1571797	1243903	10741290
HPD95Lo	318493 ?	796233	567922	0
HPD95Hi	1359589 ?	2468607	2017694	43807078

RUN3 seed 9101 gen 1942

Value	t0	q0	q1	q2
HiSmth	775114	1424087	1087329	371005
Mean	812501	1582518	1246554	10800899
HPD95Lo	316210 ?	784817	579338	0
HPD95Hi	1341324 ?	2491438	2017694	43864155

RUN4 seed 9101 gen7538

Value	t0	q0	q1	q2
HiSmth	765982	1418379	1098744	371005
Mean	810197	1583137	1252336	10717596
HPD95Lo	313927 ?	779110	573630	0
HPD95Hi	1339041 ?	2485731	2023402	43750000

CO1	t	q0	q1	q2
HiSmth	788813	1406963	1110160	313927
	713470	1401256	1098744	371005
	775114	1424087	1087329	371005
	765982	1418379	1098744	371005
mean	760844.75	1412671.25	1098744	356735.5

16s+CO1

RUN1 seed 1234 gen 13744

Value	t0	q0	q1	q2
HiSmth	685482	1195242	1001663	330136
Mean	742876	1322251	1128173	11820723
HPD95Lo	298923	677529	542473	0
HPD95Hi	1211299	2050595	1798491	46729252

RUN2 seed 5678 gen 12043

Value	t0	q0	q1	q2
HiSmth	666275	1181737	1001663	330136
Mean	741955	1317160	1129388	11774379
HPD95Lo	301324 ?	682031	533470	0
HPD95Hi	1211299 ?	2046093	1807495	46669228

RUN3 seed 9101 gen 13674

Value	t0	q0	q1	q2
HiSmth	735903	1186239	1006165	330136
Mean	741109	1317394	1132546	11802783
HPD95Lo	298923 ?	677529	542473	0
HPD95Hi	1213700 ?	2037089	1811997	46669228

16s+CO1	t	q0	q1	q2
	685482	1195242	1001663	330136
	666275	1181737	1001663	330136
	735903	1186239	1006165	330136
mean	695886.667	1187739	1003164	330136

Chapter 4
Integrating southern African species into a world phylogeny of *Siphonaria* species, and an analysis of biogeographic patterns in the genus.

ABSTRACT

This chapter integrates the southern African species of *Siphonaria* into the most recent species-level molecular phylogeny of the genus (Dayrat *et al.* 2014), which focused strongly on the Indo-west Pacific species but omitted all South African species. For this study, specimens of representative species were obtained from across the global distribution of *Siphonaria* including South Africa, Australia, New Zealand, south-east Asia, South and North America, and sequenced for the mitochondrially-encoded COI and 16S DNA regions. Additional sequences available in GenBank including those from Dayrat *et al.* 2014 (152 sequences) were incorporated in the phylogeny. I also explored biogeographic patterns such as species richness of the genus in relation to latitude, and the effect of direct vs planktonic development on the geographic ranges of species.

I sequenced 120 individuals collected from 51 localities including 11 southern African species, three of which I consider to be undescribed species, and 42 other species from around the world, nine of which were not previously included in any published phylogeny, thus adding a total of 20 species to the global phylogeny. In total, I analysed 272 mtDNA sequences (16S + COI, 1202 nucleotide characters) within a Bayesian framework, using sacoglossans as the outgroup as per Bouchet *et al.* (2017).

Similar to the phylogeny of Dayrat *et al*. (2014), two major clades, A and B, were recovered. The inclusion of the South African species led to significant rearrangement of the tree topology with the position of the South African species clear and distinct while maintaining the original division into two major clades. The South African species are not monophyletic; all of the South African members appeared in the clade A with the exception of the highly divergent *S. serrata* which occurred in clade B. Within clade A all bar two of the South African species were retrieved in one clade. The exceptions were *S. compressa* and its sister *Siphonaria* nov. sp. 3 which fell elsewhere within clade A and were more closely related to other Pacific Ocean species than to the other South African species.

The contents of the clade B - within which *S. serrata* appeared - remained similar to that in the phylogeny of Dayrat *et al.* (2014), with the exception of the rearrangement of the basal species. I was able to attach species names to six of hitherto unidentified units in that phylogeny, but 23 remain unnamed, although I too recovered them as genetically discrete entities that are likely to be distinct species.

There were strong latitudinal trends evident in the species richness with high diversity in the lower latitudes, but with a marked dip at the equator, which is possibly attributable to the fact that the limpet shell form is ill adapted to thermal and desiccation stress. Direct developers had significantly smaller geographic and latitudinal ranges per species than species with planktonic development, as would be predicted from the longer pelagic dispersal phase of the latter.

4.1 INTRODUCTION

Siphonariids are a globally distributed family of marine intertidal pulmonate limpets, found in sub-Antarctic, temperate and tropical regions of the world (Hodgson 1999). They typically have an asymmetrical patelliform shell with a distinct siphonal groove extending from the lung on the right, which interrupts the horseshoe-shaped muscle scar (White and Dayrat 2012), often providing the easiest diagnostic feature that can be used to identify shells of fossilized members of the family. The genus extends back to the Cretaceous (Dayrat *et al.* 2011), with a major Eocene diversification (Le Renard and Pacaud 1995, Harzhauser *et al*. 2017).

Until recently there has been confusion regarding their phylogenetic placement (Haszprunar 1988, Hodgson 1999, Dayrat *et al.* 2011). Traditionally they have been considered the most primitive pulmonates, with a lung-like pulmonary cavity containing a secondary gill similar to a ctenidium, which was initially regarded as homologous to the pallial cavity of non-pulmonate gastropods (Lindberg and Ponder 2001). However, Siphonariidae lack two key pulmonate features: a contractile pneumostome and pulmonary blood vessels. They do, however, have an osphradium and pallial raphes (ciliary tracts) (Mordan and Wade 2008). On the grounds that the pneumostome of *Siphonaria* spp. is not contractile – in contrast to the situation in all other pulmonates – Dayrat *et al.* (2011) propose that it is not homologous to that of other pulmonates, and that the gills of *Siphonaria* and cephalaspideans (shelled sacoglossans) are anatomically comparable.

In analyses of complete mitochondrial genomes *Siphonaria* nests within the monophyletic opisthobranchs (White *et al.* 2011). Supporting this, the mitochondrial gene order of *Siphonaria gigas* resembles that of the opisthobranchs not the pulmonates. Specifically, the genus *Siphonaria* appeared to be most closely related to opisthobranch sacoglossans and individual gene analyses suggest they could be at the base of the monophyletic Pulmonata, as it was referred to at the time (White *et al.* 2011).

In some analyses (Jörger *et al.* 2010, Medina *et al.* 2011) the Siphonariidae cluster together with the Sacoglossa. In others – as in more recent molecular work (Jörger *et al.* 2014, Zapata *et al.* 2014, Teasdale 2017) – Sacoglossa have been recovered as the sister taxon to all other panpulmonates (Zapata *et al.* 2014, Schrödel 2014, Teasdale 2017). These latter analyses are referenced as the current taxonomic status of *Siphonaria* in the most recent taxonomic revision of the Gastropoda in Bouchet *et al.* (2017). According to this, the *Siphonaria* fall within the Panpulmonata and are recognized as sharing a more recent common ancestor with pulmonates than with opisthobranchs. The full classification of the family is thus Class Gastropoda, Subclass Heterobranchia, Grade "Lower Heterobranchia", Infraclass Euthyneura, Cohort Tectipleura and Subcohort Panpulmonata. Nested within the latter are six Superorders: Sacoglossa, Siphonarimorpha, Pylopulmonata, Acochlidimorpha, Hygrophila and Eupulmonata. The Family Siphonariidae Gray 1827 (marine pulmonate 'false limpets'), which is dominated

by the genus *Siphonaria* Sowerby, 1823, falls within the Superorder Siphonarimorpha, Order Siphonariida, Superfamily Siphonarioidea. The type species of the genus is *S. sipho* Sowerby, 1823, which has since been synonymized with *S. javanica* (Lamarck, 1819).

In the most recent published siphonariid phylogeny, Dayrat *et al*. 2014 recognised 41 distinct species or molecular units (or Evolutionary Significant Units) after application of their two criteria, namely, reciprocal monophyly and genetic distance. They placed emphasis on samples from the tropical and subtropical Indo-West Pacific and no southern African species were represented. An inclusive phylogeny is thus necessary to place the southern African species within a global context together with other species and the 41 units already recognised. This is the primary aim of this chapter. This phylogeny will also establish whether the South African species of *Siphonaria* are monophyletic. A second aim of this chapter is to explore phylogeographic patterns in the distributions of *Siphonaria* species. In addressing these goals, I drew on available genetic information in GenBank as well as sequencing that I undertook for a range of species gathered from around the world, combined with geographic distributional records in the literature or in relevant websites.

I hypothesized that:
(1) the southern African species would form a discrete and monophyletic group based on their distinct geographic location (Chapter 2)
(2) the major clades emerging from the phylogenetic analysis would have distinctive geographical distributions.
(3) species richness within the genus would display latitudinal gradients, declining towards higher latitudes in accordance with patterns displayed by many marine taxa (Willig and Presley 2018), but with a dip in the tropics based on the argument that the limpet shell form is ill adapted for desiccation and thermal stress (Vermeij 2017).
(4) Species with direct development would have smaller geographic ranges than those with planktonic development, because of their reduced capacity for dispersal (Chambers and McQuaid 1994b, Peron and Kohn 1985).

4.2 MATERIALS AND METHODS

Sample collection

Between 2005 and 2011,128 specimens of *Siphonaria* specimens were collected by myself or supplied to me by other researchers from southern Africa and around the world. All material was preserved in ~95% ethanol. An additional nine samples were obtained from The Natural History Museum, London. Collection locality, species names (provisionally identified in some cases by the collectors) and names of collectors or

Table 4.1A GenBank sequences not present in Dayrat et al 2014 added to the phylogeny.

GenBank sequences	16S	COI
S. fuegiensis	na	MF652008
S. diemenensis	na	JX680931
S. funiculata	na	JX 680959
S. tasmanica	na	JX680964
S. brannani	KM204294	KM086435
S. denticulata	na	JX680899

Table 4.1B Sample identifiers, collection localities, names of collectors, provisional (field) identifications, and final names allocated to all new material employed in phylogeny. BMNH: Natural History Museum, London. Unnamed and new species in bold.

Sample name	Collection locality	Latitude	Longitude	Collector	Field identity	Species name
thersites351tatoosh	Tatoosh Island, Clallam, Washington State, USA	48.389369	-124.734664	R.T. Paine	*S. thersites*	*S. thersites*
Kerg383TdFBM	**Tierra del Fuego, Argentina**	**-54.830687**	**-68.292571**	**BMNH**	***Kerguelenella lateralis***	***unnamed***
Kerg384	Tierra del Fuego, Argentina	-54.830687	-68.292571	BMNH	*Kerguelenella lateralis*	*S. fuegiensis*
jean142Rtn	Cape Vlamingh, Rottnest Island, W Australia	-32.019035	115.494768	R. Black	*S. jeanae*	*S. jeanae*
SEAus399PmblaB	Pambula Beach, SE Australia	-36.946088	149.915066	T.Nakano	no identity provided	*diemenensis*
TA2Tas	Tasmania, Australia	-41.27671	148.3481	S. Ling	no identity provided	*S. diemenensis*
TA1_443Tas	Tasmania, Australia	-42.901095	147.366708	S. Ling	no identity provided	*S. diemenensis*
virg343Sydney	Sydney Harbour, Australia	-33.870733	151.255347	J. Moreira	*S. virgulata*	*S. funiculata*
TB_445_6Tas	Tasmania, Australia	-42.895121	147.358553	S. Ling	no identity provided	*S. funiculata*
virg344Sydney	Sydney Harbour, Australia	-33.870733	151.255347	J. Moreira	*S. virgulata*	*S. funiculata*
less5Chile	Quintay, Chile	-33.193479	-71.700571	T. Manzur	*S. lessonii*	*S. lessonii*
less6Chile	Quintay, Chile	-33.193479	-71.700571	T. Manzur	*S. lessonii*	*S. lessonii*
con28SBR	Stillbaai, South Africa	-34.390498	21.411485	L. Kemp	*S. concinna*	*S. concinna*
con32	Stillbaai, South Africa	-34.390498	21.411485	L. Kemp	*S. concinna*	*S. concinna*
oculus439Mkb	Mkambati, South Africa	-31.319353	29.972663	M. Pfaff	*S. oculus*	*S. oculus*
oculus183RR	Rabbit Rock, South Africa	-27.035786	32.86031	J. Harris	*S. oculus*	*S. oculus*
oculus153Stnga	Stanga, South Africa	-29.383360	31.343179	C. Moloney	*S. oculus*	*S. oculus*
oculus152Bal	Ballito, South Africa	-29.533521	31.223834	C. Moloney	*S. oculus*	*S. oculus*
oculus151BhN	Bhanga Neck, South Africa	-27.012069	32.865892	J. Harris	*S. oculus*	*S. oculus*
oculus118BhN	Bhanga Neck, South Africa	-27.012069	32.865892	J. Harris	*S. oculus*	*S. oculus*
Dbn182	Durban, South Africa	-29.875401	31.061651	C. Moloney	query	*S. anneae*
QMoz429Mkb	**Mkambati, South Africa**	**-31.319353**	**29.972663**	**M. Pfaff**	**query**	**new species 2**
Q181Mkb	**Mkambati, South Africa**	**-31.319353**	**29.972663**	**M. Pfaff**	**query**	**new species 2**
QMoz368XX	Pariso de Chidenguele, Mozambique	-25.115072	33.746061	G. Branch	query	*S. tenuicostulata*
QMoz369XX	Pariso de Chidenguele, Mozambique	-25.115072	33.746061	G. Branch	query	*S. tenuicostulata*
Q112Tofo	**Tofo, Mozambique**	**-23.850866**	**35.543631**	**L. Kemp**	**query**	**new species 1**
Moz115Tofo	**Tofo, Mozambique**	**-23.850866**	**35.543631**	**L. Kemp**	**query**	**new species 1**
Moz117Tofo	**Tofo, Mozambique**	**-23.850866**	**35.543631**	**L. Kemp**	**query**	**new species 1**
Qcap329BhN	Bhanga Neck, South Africa	-27.012069	32.865892	J. Harris	query	*S. nigerrima*
nig11_2RR	Rabbit Rock, South Africa	-27.035786	32.86031	J. Harris	*S. nigerrima*	*S. nigerrima*
Qcap330BhN	Bhanga Neck, South Africa	-27.012069	32.865892	J. Harris	query	*S. nigerrima*
ann89_90RR	Rabbit Rock, South Africa	-27.035786	32.86031	J. Harris	*S. anneae*	*S. nigerrima*
ten10BhN	Bhanga Neck, South Africa	-27.012069	32.865892	J. Harris	*S. tenuicostulata*	*S. nigerrima*
nig93_4BhN	Bhanga Neck, South Africa	-27.012069	32.865892	J. Harris	*S. nigerrima*	*S. nigerrima*
ten9BhN	Bhanga Neck, South Africa	-27.012069	32.865892	J. Harris	*S. tenuicostulata*	*S. nigerrima*
cap175EB	Eland's Bay, South Africa	-32.313083	18.337995	P. de Coito	*S. capensis*	*S. capensis*
pec371GCanarias	Gran Canaria, Spain	28.142078	-15.435695	G. Branch	*S. pectinata*	*S. pectinata*
pec339Prtgl	Paimogo, Portugal	39.285405	-9.337641	A. Silva	*S. pectinata*	*S. pectinata*
Port30Prtgl	Portugal	38.703516	-9.397127	B. Xavier	*S. pectinata*	*S. pectinata*
Port31Prtgl	Portugal	38.703516	-9.397127	B. Xavier	*S. pectinata*	*S. pectinata*
pec370GCanarias	Gran Canaria, Spain	28.142078	-15.435695	G. Branch	*S. pectinata*	*S. pectinata*
pec380GhanaBM	Old Ningo, Accra, Ghana	5.744895	0.193542	BMNH	*S. pectinata*	*S. pectinata*
pec379GhanaBM	Old Ningo, Accra, Ghana	5.744895	0.193542	BMNH	*S. pectinata*	*S. pectinata*
HK418HKc	Hong Kong	22.231117	114.168798	G. Williams	*S. japonica*	*S. japonica*
Taiwan373PngHu	Peng-hu, Taiwan	23.582446	119.582844	T. Nakano	no identity provided	*S. japonica*
japon392HK	Hong Kong	22.231117	114.168798	B. Chan	*S. japonica*	*S. japonica*
HK417HKc	Hong Kong	22.231117	114.168798	G. Williams	*S. japonica*	*S. japonica*
japon391HK	South Bay, Hong Kong	22.225850	114.197961	B. Chan	*S. japonica*	*S. japonica*
comK2Knysna	**Knysna, South Africa**	**-34.049309**	**23.046499**	**P. de Coito**	**query**	**new species 3**
comL7Langebaan	Langebaan, South Africa	-33.137480	18.077134	P. de Coito	*S. compressa*	*S. compressa*
norm342Oahu	NE side of Coconut Island, 'Oahu, Hawaii	21.496298	-157.845795	M. Craig, C. Bird	*S. normalis*	*S. normalis*
norm341Oahu	NE side of Coconut Island, 'Oahu, Hawaii	21.496298	-157.845795	M. Craig, C. Bird	*S. normalis*	*S. normalis*

1

Marqu385MarquBM	Taihoae Bay, Nuku Hiva, Marquesas Islands	-8.840004	-140.185258	BMNH	no identity provided	unit 14
NZ440Chrch	Sumner, Christchurch, New Zealand	-43.565502	172.754645	H. Spencer	no identity provided	*S. australis*
aus111NZ	Brighton Beach, Dunedin, New Zealand	-45.946687	170.333576	J. Waters, C. Fraser	*S. australis*	*S. australis*
NZ400Chrstch	Sumner, Christchurch, New Zealand	-43.565502	172.754645	H. Spencer	no identity provided	*S. australis*
aus109NZ	St Claire Beach, Dunedin, New Zealand	-45.911995	170.491471	H. Spencer, T. King	*S. australis*	*S. australis*
NZ402Chrch	Sumner, Christchurch, New Zealand	-43.565502	172.754645	H. Spencer	no identity provided	*S. australis*
Saus110NZ	Brighton Beach, Dunedin, New Zealand	-45.946687	170.333576	J. Waters, C. Fraser	*S. australis*	*S. australis*
gigas334Panama	Punta del Culebra, Panama City	8.910708	-79.528792	A. Hodgson	*S. gigas*	*S. gigas*
gigas333Panama	Punta del Culebra, Panama City	8.910708	-79.528792	A. Hodgson	*S. gigas*	*S. gigas*
Moz378PembaBayBM	Pemba Bay, Mozambique	-12.940229	40.504379	BMNH	no identity provided	*S. kurracheensis*
RedSea338RdS	Eilat, Red Sea	29.544990	34.951776	Y. Achitov	*S. crenata or savignyi*	*S. belcheri*
RedSea337RdS	Eilat, Red Sea	29.544990	34.951776	Y. Achitov	*S. crenata or savignyi*	*S. belcheri*
Taiwan372PngHu	**Peng-hu, Taiwan**	**23.582446**	**119.582844**	**T. Nakano**	**no identity provided**	**unnamed a**
Japan403Ogswra	**Islands**	**27.095749**	**142.202257**	**T. Nakano**	**no identity provided**	**unnamed a**
Japan404Ogswra	**Islands**	**27.095749**	**142.202257**	**T. Nakano**	**no identity provided**	**unnamed a**
JapanE1403077	**Islands**	**27.095749**	**142.202257**	**T. Nakano**	**no identity provided**	**unnamed a**
ser172Mkb	Mkambati, South Africa	-31.273006	30.028108	M. Pfaff	*S. serrata*	*S. serrata*
ser173CVd	Cape Vidal, South Africa	-28.147184	32.555564	J. Harris	*S. serrata*	*S. serrata*
ser162BB	Betty's Bay, South Africa	-34.360607	18.952348	P. de Coito	*S. serrata*	*S. serrata*
ser161Hnk	Hangklip, South Africa	-34.337792	18.826213	P. de Coito	*S. serrata*	*S. serrata*
Japan389Kgshma	Kagoshima, (Hakagoshi) Japan	31.539069	130.547262	T. Nakano	no identity provided	*S. sirius*
HK406HKa	Hong Kong	22.231117	114.168798	G. Williams	*S. sirius/atra*	*S. sirius*
HK415HKb	Hong Kong	22.231117	114.168798	G. Williams	*S. sirius/atra*	*S. sirius*
HK416HKb	Hong Kong	22.231117	114.168798	G. Williams	*S. sirius/atra*	*S. sirius*
Japan390Kgshma	Kagoshima, (Hakagoshi) Japan	31.539069	130.547262	T. Nakano	no identity provided	*S. sirius*
HK405HKa	Hong Kong	22.231117	114.168798	G. Williams	*S. sirius/atra*	*S. sirius*
den345Sydney	Sydney, Australia	-33.870733	151.255347	J. Moreira	*S. denticulata*	*S. denticulata*
Tanz388TanzBM	Zanzibar, Tanzania	-5.921575	39.202395	BMNH	no identity provided	unit 35
Tanz375DeSBM	Dar es Salaam, Tanzania	-6.718626	39.225592	BMNH	no identity provided	unit 35
Tanz387TanzBM	Zanzibar, Tanzania	-5.921575	39.202395	BMNH	no identity provided	unit 35
Indns377BBBM	Buton Island, Sulawesi, Indonesia	-5.454673	122.604072	BMNH	cf. *S. javanica* (on label)	unit 39
Japan420SonaiB	Sonai, Yonaguni Island, Japan	24.471757	123.002137	T. Nakano	no identity provided	unit 39
Japan408SonaiA	Sonai, Yonaguni Island, Japan	24.471757	123.002137	T. Nakano	no identity provided	unit 39
kur145Rtn	**Cape Vlamingh, Rottnest Island, W Australia**	**-32.019035**	**115.494768**	**R. Black**	***S. kurracheensis***	**unnamed b**
kur146Rtn	**Cape Vlamingh, Rottnest Island, W Australia**	**-32.019035**	**115.494768**	**R. Black**	***S. kurracheensis***	**unnamed b**
kur147Rtn	**Cape Vlamingh, Rottnest Island, W Australia**	**-32.019035**	**115.494768**	**R. Black**	***S. kurracheensis***	**unnamed b**
zel140Pth	Trigg Beach, Perth, W Australia	-31.975088	115.758499	R. Black	*S. zelandica*	*S. zelandica*
zel139Pth	Trigg Beach, Perth, W Australia	-31.975088	115.758499	R. Black	*S. zelandica*	*S. zelandica*
den346Sydney	Sydney, Australia	-33.870733	151.255347	J. Moreira	*S. denticulata*	*S. zelandica*
Japan395Wkym	Kushimoto, Wakayama, Japan	33.488647	135.798942	T. Nakano	no identity provided	*S. zelandica*
JapanC	Kushimoto, Wakayama, Japan	33.488647	135.798942	T. Nakano	no identity provided	*S. zelandica*
Japan396Wkym	Kushimoto, Wakayoma, Japan	33.488647	135.798942	T. Nakano	no identity provided	*S. zelandica*
kur148Ning	Ningaloo, NW Australia	-21.916742	113.973565	R. Bustamante	*S. kurracheensis*	*S. javanica*
kur150Ning	Ningaloo, NW Australia	-21.916742	113.973565	R. Bustamante	*S. kurracheensis*	*S. javanica*
kur149Ning	Ningaloo, NW Australia	-21.916742	113.973565	R. Bustamante	*S. kurracheensis*	*S. javanica*
EastTimor382	Dili Bay, East Timor	-8.549923	125.571288	BMNH	no identity provided	*S. javanica*
ETimor381DBBM	Dili Bay, East Timor	-8.549923	125.571288	BMNH	no identity provided	*S. javanica*
Bru347Brunei	Darussalam, Brunei	4.951168	115.034978	D.J. Marshall	no identity provided	*S. javanica*
Bru348Brunei	Darussalam, Brunei	4.951168	115.034978	D.J. Marshall	no identity provided	*S. javanica*
ETimor376JBayBM	Jaco Island, East Timor	-8.416748	127.309116	BMNH	no identity provided	*S. javanica*
Japan407SonaiA	Sonai, Yonaguni Island, Japan	24.471757	123.002137	T. Nakano	no identity provided	*S. lacionosa*
Taiwan397Jlshui	Jialeshui, Taiwan	22.006304	120.877464	B. Chan	*S. laciniosa*	*S. lacionosa*
Taiwan398Jlshui	Jialeshui, Taiwan	22.006304	120.877464	B. Chan	*S. laciniosa*	*S. lacionosa*

museums are shown in Table 4.1B. Additional outgroup sequences not included in Dayrat *et al.* (2014) (Table 4.1A) and all *Siphonaria* sequences referred to in the phylogeny of Dayrat *et al.* (2014) (Appendix 4.1) were obtained from GenBank.

Laboratory procedures and sequence analysis

Details of the methodology for DNA extraction, choice of markers, DNA amplification and sequencing, as well as sequence assembly, multiple sequence alignment and tree building are outlined in Chapter 2, except for aspects that depart from those procedures, as outlined below.

Phylogenetic analysis

After alignment in Jalview (Waterhouse *et al.* 2009) the ends of the two separate alignments (16S and COI) were trimmed and concatenated in Seaview (Gouy *et al.*

2010). All substitution model selection testing was done in Topali v2.5 (Milne *et al.* 2009) which offers the models available in the programs MrBayes and PhyML (Guindon *et al*. 2010), and was computed remotely via The James Hutton Institute, Scotland (https://www.hutton.ac.uk). Hierarchical likelihood ratio tests, the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC) were used to compare possible models. Models with the lowest AIC and BIC scores are thought to describe the most suitable substitution model for the dataset (Bofkin and Goldman 2006). Among-site rate variation was addressed by partitioning the data into coding (COI) and non-coding (16S) portions and generating specific models for each.

A concatenated 16S and COI dataset consisting of 272 sequences, which included both my sequences (Table 4.1) and the 152 sequences referred to in Dayrat *et al.* (2014) (COI: KF000688-839, 16S: KF000840-991)(Appendix 4.1) were used to generate a Bayesian tree with Mr Bayes v3.2.6x64 (with Beagle set to active) (Ronquist *et al.* 2012), which was computed remotely via the Cipres Science Gateway v3.3 (Miller *et al.* 2010). I was guided in my choice of outgroups by the phylogenetic work of Zapata *et al.* (2014) and Teasdale (2017) in which the members of the superorder Sacoglossa (sap-sucking sea slugs) were recovered as sister to all other panpulmonates. I selected two shelled Oxynooidea (*Oxynoe antillarum*, *Volvatella viridis*) and four shell-less plakobranchoidean sacoglossans (*Bosellia mimetica*, *Cyerce nigricans*, *Elysia viridis*, *Plakobranchus ocellatus*) as outgroups for my concatenated 16S plus COI analyses. The final sequence length after adjustments as a result of concatenation was 1202 bases.

The Generalised time-reversible with gamma distributed rate variation among sites (GTR+G) (Tavaré 1986) (with the lowest BIC of 61199.70, lowest AIC = 58410.77) was chosen as the 16S partition model, and GTR+I+G, i.e. with a proportion of invariable sites, (lowest BIC of 83729.51, lowest AIC = 80902.40) was the chosen model for the COI portion. Both PhyML and MrBayes generated the lowest BIC and AIC scores for these substitution models for these data.

The optimised conditions for the final Bayesian analyses were run for 140 million generations sampling every 100,000th generation, using 4 chains and with the temperature set at 0.05. The first 25% of trees generated were discarded as burnin. The final runs showed acceptance rates of 12-40% and chain swapping proportions of 15-35%. The total number of trees sampled was 2102. The potential scale reduction factor (PSRF) tended to 1; evolutionarily stable strategy (ESS) values were all greater than 200; and the average standard deviation of split frequencies (ASDSF) value was 0.02.

Trace plots of Log likelihood vs generation number made in Tracer (Rambaut *et al.* 2018) were used to infer mixing behaviour and thereby monitor the Markov chain Monte Carlo (MCMC) runs. Once conditions were optimised, runs were repeated with the same conditions but with different starting seeds to improve the probability that optimal trees were being sampled. All saved trees were summarised using a majority-rule consensus and is the tree with the maximum product of posterior clade probabilities where the posterior probabilities (clade credibilities) calculated for each branch split are interpreted as node support. The tree was visualised in Figtree v1.4.0 (Rambaut 2016).

The sequences produced for the analysis for this chapter will be submitted to GenBank. The shells and tissues of all specimens will be deposited with the KwaZulu-Natal Museum, Pietermaritzburg, South Africa.

Geographic analyses

All currently recognized species in the genus *Siphonaria* were tabulated and their distribution determined from records in the literature (Appendix 4.2A, B). I evaluated relationships between the clades evident from my analysis and their geographic distribution, and plotted the numbers of species occurring in 10° latitudinal bands. A chi-squared analysis was applied to test for the significance of departure from the null hypothesis that species richness is independent of latitude.

I tabulated information on larval development types drawn from the literature (Table 4.2) and tested whether the geographic ranges of species were greater for those with planktonic larvae than for direct developers, using a one-tailed t-test assuming unequal variance due to inequalities of variance (with according reductions in degrees of freedom), as there was a significant difference in variance. Range was defined in two ways: (a) the greatest latitudinal range; and (b) as the maximal linear distance measured across the spread of each species. As there was one outlier in the data for maximal linear distance (*S. lateralis*) I ran the test both including and excluding it. Following the classification of Chambers and McQuaid (1994a, b) larval developmental types were classed as planktonic, direct, or 'swimming/crawling', but as there were only three cases of the latter I excluded them from statistical analysis.

I also tested if the latitudinal range of species with planktonic development was greater than that for direct developers, using a t-test for equal variance after verifying with Shapiro-Wilk and Levene's tests that there were respectively no significant differences in normality and variance.

Table 4.2 Distribution, larval development, sources of information and maximum geographic and latitudinal range for species for which larval development type is known. Names in bold were included in my phylogeny**.**

Species	Distribution	Larval development	References for larval type	Max. geogr. Range (km)	Max. Latitud. Range (km)
Siphonaria alternata (Say, 1826)	East Florida; E&W coasts of Panama; Bahamas; Bermuda	Swimming/crawling	Zischke 1974	7548	7548
Siphonaria anneae Tomlin, 1944	KwaZulu-Natal, South Africa	Direct	Chambers & McQuaid 1994a,b	22	55
Siphonaria atra Quoy & Gaimard, 1833	Solomon Islands, Philippines; Japan. Vanikoro Island	Planktonic	Abe 1941	6882	3885
Siphonaria australis Quoy & Gaimard, 1833	New Zealand	Planktonic	Russell & Phillips 2009; Fitzpatrick et al. 2010	2442	1887
Siphonaria capensis Quoy & Gaimard, 1833	Angola to East Coast of South Africa	Planktonic	Chambers & McQuaid 1994a,b	2775	2664
Siphonaria compressa Allanson, 1958	Langebaan, South Africa	Direct	Chambers & McQuaid 1994a,b	22	11
Siphonaria concinna G. B. Sowerby I, 1823	South-east South Africa	Planktonic	Chambers & McQuaid 1994a,b	1998	1665
Siphonaria denticulata Quoy & Gaimard, 1833	Port Western; Victoria, W Australia, Queensland	Planktonic	Creese 1980	6108	3330
Siphonaria diemenensis Quoy & Gaimard, 1833	Tasmania, S Australia, NSW	Planktonic	Mapstone 1978	2331	1554
Siphonaria funiculata Reeve, 1856	Tasmania, NSW, Queensland, W Victoria	Planktonic	Creese 1980	2997	2997
Siphonaria gigas G. B. Sowerby I, 1825	Panama Pacific Coast	Planktonic	Levings & Garrity 1986	1776	1100
Siphonaria guamensis Quoy & Gaimard, 1833	Guam, Billiton, Java Sea, Singapore	Swimming/crawling	Chim & Tan 2009	4218	4218
Siphonaria hispida Hubendick, 1946	Rio de Janeiro, S Brazil; Isle Fernando	Swimming/crawling	Marcus & Marcus 1960	2664	1100
Siphonaria japonica (Donovan, 1824)	Japan; Hong Kong	Planktonic	Abe 1940, Huang & Chan 2000	3996	3663
Siphonaria javanica (Lamarck, 1819)	Indonesia, Thailand, Philippines	Planktonic	Thorson 1940	3885	2443
Siphonaria kurracheensis Reeve, 1856	Karachi Pakistan, also Red Sea, N Mozambique	Direct	Thorson 1940	3663	3441
Siphonaria lateralis Gould, 1846	Tierra del Fuego; Falklands, sub-Antarcric Islands	Direct	Simpson 1977, Simpson & Harrington 1985	23310	555
Siphonaria lessonii Blainville, 1827	Falkland Islands, Argentina, Chile, Punta del Esta Uruguay	Planktonic	Berry 1977, Zabala et al. 2018	2997	2553
Siphonaria macquariensis (Powell, 1939) *	Macquarie Island, Australia	Direct	Knox 1955	888	333
Siphonaria naufragum Stearns, 1872	Florida, Gulf of Mexico	Planktonic	Voss 1959, Zischke 1974	1998	1665
Siphonaria nigerrima Smith, 1903	Umhlali to Durban, KwaZulu-Natal, South Africa	Direct	Chambers & McQuaid 1994a,b	66	100
Siphonaria oculus F. Krauss, 1848	SE coast of South Africa	Planktonic	Chambers & McQuaid 1994a,b	1998	1100
Siphonaria pectinata (Linnaeus, 1758)	Mediterranean, Canary Islands, West Africa	Planktonic	Slama et al. 2018; Ocaña & Emson 1999	6438	4995
Siphonaria placentula Menke, 1853*	Cape Verde Islands	Planktonic	Infered from relationship witrh *S. pectinata*	555	450
Siphonaria serrata (Fischer von Waldheim, 1807)	SE Coast of South Africa	Direct	Chambers & McQuaid 1994a	1332	777
Siphonaria stewartiana (Powell, 1939)	Stewart Island, South Island, New Zealand.	Direct	Knox 1955	222	50
Siphonaria tasmanica Tenison Woods, 1876	Tasmania, Victoria, New South Wales, Queensland, W Australia	Planktonic	Quinn 1988	3885	2777
Siphonaria tenuicostulata Smith, 1903	Chidinguele, Mozambique	Direct	Chambers & McQuaid 1994a	111	111
Siphonaria zelandica Quoy & Gaimard, 1833	New Zealand & Keppel Bay to Broome, E Australia	Planktonic	Mapstone 1978	4440	3662
New species 3	Knysna Lagoon, South Africa	Direct	Allanson & Msizi 2010	16	5

*Excluded from analysis due to doubts about geographic range

4.3 RESULTS

Siphonaria was resolved as monophyletic with maximal support (Bayesian Probability = 1). Within *Siphonaria* two distinct clades were resolved, clade A with maximal support (BP = 1) and a less well supported (BP = 0.75) clade B (Fig. 4.1). Figs 4.2A and 4.2B show relationships within these clades separately and the intra-clade relationships and relationships with the 41 genetic units as designated by Dayrat *et al.* (2014)(Appendix 4.1).

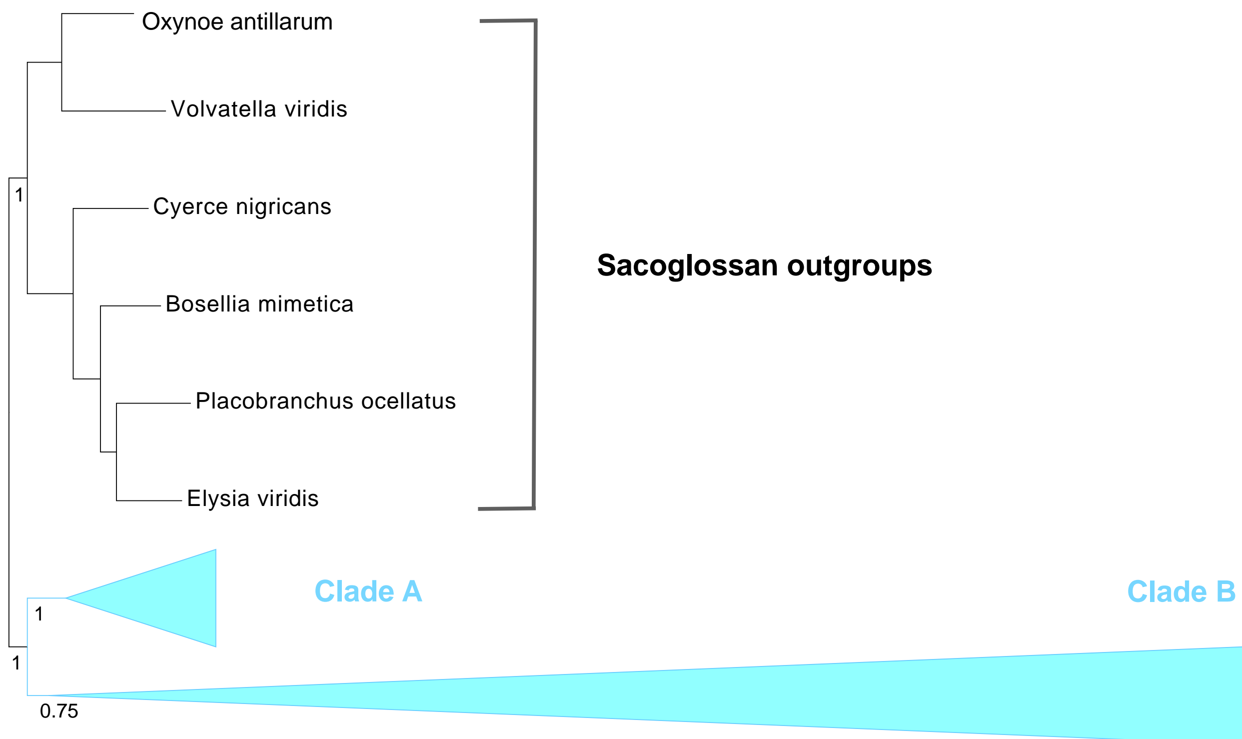

Figure 4.1 Phylogenetic relations of *Siphonaria* in relation to the sacoglossan outgroup.

Sacoglossan outgroups

CLADE A

A1

ther351Tatoosh
Kerg383TdFBM
Kerg383
S. thersites
unnamed
MLP13163
MLP13163b
BMNH1994092
6
S. lateralis
Siphonaria fuegiensis
Kerg384
MLP13170b
MLP13170
5
S. fuegiensis

a

i

jean142Rtn
S. jeanae
SEAus399PmblaB
CASIZ189427
CASIZ189428
Siphonaria diemenensis
TA2Tas
TA1Tas
7
S. diemenensis

α

AMC395918
virg343Sydney
TBTas
virg344Sydney
UF351617
UF351837
BMNH20080193
Siphonaria funiculata
8
S. funiculata
Siphonaria tasmanica
S. tasmanica
less5Chile
less6Chile
Chile
MLP13166
MLP13168
MLP13164
MLP13165
MLP13169
MLP13167
Argentina
9
S. lessonii

ii

β

con28SBR
con32
S. concinna
oculus439Mkb
oculus183RR
oculus153Stnga
oculus152Bal
oculus151BhN
oculus118BhN
S. oculus
3 Oman
S. ashgar
S. anneae
Dbn182
QMoz429Mkb
Q181Mkb
new species #2 Mkambati
QMoz368XX
QMoz369XX
S. tenuicostulata
Q112Tofo
Moz115Tofo
Moz117Tofo
new species #1 Tofo
Qcap329BhN
nigRR
Qcap330BhN
annRR
ten10BhN
nigBhN
ten9BhN
S. nigerrima
cap175EB
S. capensis
UF382817
UF254828
UF79313
UF54116
S.naufragum
Americas
pec371GCanarias
pec339Prtgl
Port30Prtgl
Port31Prtgl
pec370GCanarias
pec380GhanaBM
pec379GhanaBM
BMNH20080071
S. pectinata
Africa and Europe
4

A2

b

HK418HKc
Taiwan373PngHu
japon392HK
HK417HKc
japon391HK
UF368383
UF350553
UF310519
CASIZ189437
UF310521
2
S. japonica
UF367730
10 Oman
comK2K
comL7L
new species #3 Knysna
S. compressa
UF310368
11 Caroline Is
norm342Oahu
norm341Oahu
S. normalis
BMNH20080191
BMNH20080194
12
UF292269
UF341298
13
S. guamensis
UF301195
UF324719
UF324705
UF324705b
UF348566
UF348025
UF400391
Marqu385MarquBM
UF303670
UF292011
BMNH20080069
UF392234
UF294623
14
"normalis" group = 12-14

c

NZ440Chrch
aus111NZ
NZ400Chrstch
aus109NZ
NZ402Chrch
Saus110NZ
S. australis

CLADE B

Clade B

S. thersites
S. lateralis
S. fuegiensis
S. jeanae
S. diemenensis
S. funiculata
S. tasmanica
S. lessonii
S. concinna
S. oculus
S. asghar
S. anneae
New sp. 2 Mkambati
S. tenuicostulata
New sp. 1 Tofo
S. nigerrima
S. capensis
S. naufragum
S. pectinata
S. japonica
10: Oman
S. compressa
11: Caroline Is
S. normalis
S. guamensis
S. australis

Figure 4A

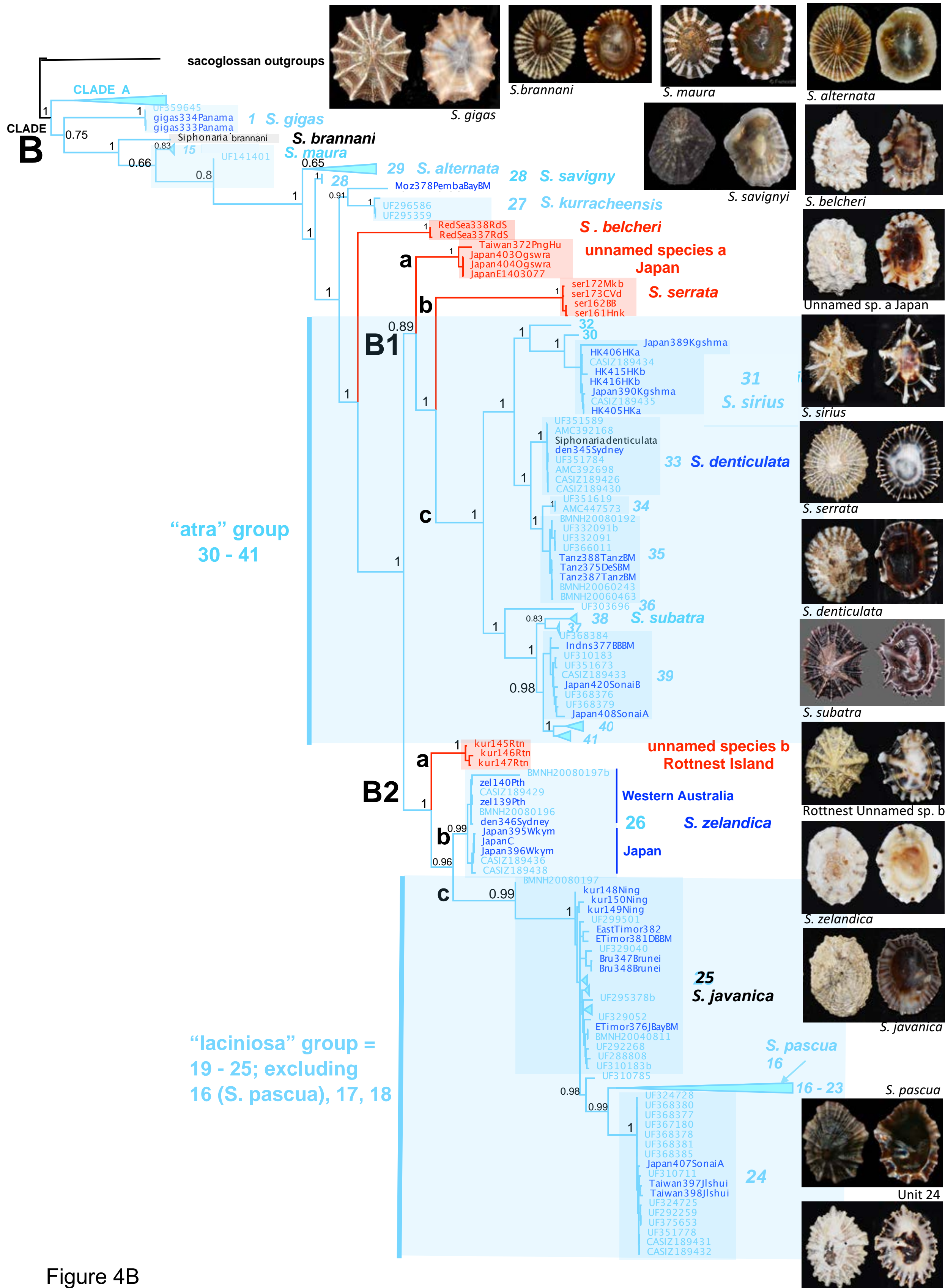

Figure 4B

Figure 4.2 Phylogenetic trees for (A) clade A and (B) clade B, obtained from concatenated 16S and COI sequences of 266 individuals of *Siphonaria*. MCMC posterior probabilities shown at nodes. Black = sequences derived from GenBank that were not included in Dayrat *et al.* (2014). Dark blue = samples sequenced by myself that fell into previously established units. Red = samples sequenced by myself that were not part of the Dayrat *et al.* phylogeny and have been added as a result of my analyses. Light blue = the 41 molecular units and individuals comprising them as described in Dayrat *et al.* (2014, Table 1; see Appendix 4.1). Units for which I had no additional samples have been collapsed and numbered according to their unit numbers. Species I consider to be 'new' in southern Africa are outlined with red boxes. Sources of photographs: all by G.M. Branch except for *S. lateralis* (Holotype #5853, US National Museum); *S. fuegiensis* Güller *et al.* (2016); S. *tasmanica* (©G & Ph Poppe, gastropods.com); *S. asghar* (type specimen, Natural History Museum, London); *S. naufragum* (Bailey-Matthews National Shell Museum, Sanibel, Florida, USA); 10 Oman and 11 Caroline Is. (Dayrat *et al.* 2014); *S. guamensis*, S. *gigas*, *S; maura, S. subatra* (Femorale.com); *S. brannani* (Specimen # 77756, Natural History Museum Rotterdam); *S. savignyi* (©David Kirsh, gastropods.com); *S. javanica* (as *S. cornuta* holotype, 5850, US National Museum); *S. pascua* (©Olivia Caro, gastropods.com).

Clade A

Within clade A (Fig. 4.2A) a small, maximally-supported (BP = 0.99) clade, A1, comprising *S thersites*, *S. fuegiensis*, *S. lateralis* and an apparently undescribed species from Kerguelen, is resolved as sister to a moderately supported clade A2 (BP = 0.71) comprising the remaining samples. The strong link between identified material of *S. fuegiensis* (GenBank ID MF652008) and specimens of "lateralis" group 5, also from Tierra del Fuego, indicates that unit 5 of Dayrat *et al.* (2014) is in fact *S. fuegiensis*, and I assigned it to that species; similarly, unit 6 corresponds with *S. lateralis*. These four taxa are therefore sisters to all other clade A members, in A2. I subsequently was able to obtain more *S. fuegiensis* and *S. lateralis* sequences from GenBank and was thus able to confirm my decision separately from the phylogeny presented here.

Within the larger clade A2 three clades, named a, b and c, with posterior probabilities 0.81, 0.87, 1.0 respectively were resolved. These formed a trichotomy. Within clade A2a, the smaller maximally-supported (BP = 0.99), clade **i**, comprising *S. jeanae* and *S. diemenensis*, formed the sister clade to the remaining samples. Identified material of *S. diemenensis* (GenBank ID JX680931) allowed me to confirm unit 7 of Dayrat *et al.* (2014) as the latter.

Within the larger, well-supported, (BP = 0.91) clade **ii**, the smaller maximally-supported (BP = 1) clade **α** comprising *S. funiculata* (unit 8), *S. tasmanica*, and *S. lessonii* (unit 9) formed the sister clade to the remaining samples. Identified material of *S. tasmanica* (GenBank ID JX680964), not previously included in any phylogeny, fell within this clade. *Siphonaria lessonii* showed evidence of geographical differentiation, as the Argentinian (BP = 0.99) and Chilean (BP = 0.74) samples separated out.

Clade **β**, the maximally-supported (BP = 1) larger sister clade to the above, diverged into a well-supported (BP = 0.82) clade comprising all the South African east coast species together with the closely-related *S. concinna* and *S. oculus* and Indo-Arabian *S. asghar* and a maximally-supported (BP = 1) clade consisting of the southern African *S. capensis* and the

widespread *S. pectinata* (unit 4) which displayed strong differentiation (BP = 1) between the American and European/West African individuals, with the American clade now recognized as a separate species, *S. naufragum* (Giribet & Kawauchi, 2016).

Within clade A2b all branches were maximally supported. *S. japonica* (unit 2) (BP = 1) is sister to a clade comprising unnamed species in units 10 (Oman) (BP = 1) and 11 (Caroline Islands) (BP = 1), the South African endemics, *S. compressa* and *Siphonaria* nov. sp. 3 Knysna (BP = 1), *S. normalis* (BP = 1) from Hawaii and the 'normalis' group (units 12-14). I provisionally identified unit 13 as *S. guamensis* (BP = 1). This is dealt with further in the Discussion.

Siphonaria australis from New Zealand was the sole taxon in clade A2c (BP = 1).

Clade B

Within clade B (Fig. 4.2B), most species were part of maximally or strongly supported clades, subtended by a grade of individually divergent species that branched successively from the base: *S. gigas* (unit 1)(BP = 0.75), *S. brannani* (GenBank ID KM204294, KM086435)(BP = 1), *S. maura* (unit 15 in which I include UF141401)(BP = 0.66), *S. alternata* (unit 29)(BP = 1), *S. savignyi* (unit 28)(BP = 1), *S. kurracheensis* (unit 27)(BP = 1), and *S. belcheri* (BP = 1), a species from the Red Sea that I sequenced, which I identified photographically to be such (Bosch *et al*. 1995, Albayrak and Çağlar 2006). There were no clear clusters of taxa until clades B1 and B2.

Clade B1 (BP = 0.89) comprising an unnamed Japanese species in B1a, the South African *S. serrata* in B1b, and a large assemblage of species in units 30-41 (B1c), that were grouped together as the "atra" group by Dayrat *et al.* (2014). There were three identifiable species within this group, *S. sirius* (unit 31), *S. denticulata* (unit 33) and *S. subatra* (unit 38). Unit 33 was previously unidentified by Dayrat *et al.* (2014) and termed part of the "atra" group, but inclusion of an identified specimen of *S. denticulata* (GenBank ID JX680899) in the unit and its restricted range in New South Wales, Australia, makes it possible to assign the name *S. denticulata* to that unit. Units 30 and 32, 34-37 and 39-41 remain unidentified but were all discrete, well supported, entities recognized previously by Dayrat *et al.* (2014) and supported by my phylogeny.

Clade B2 (BP = 1) contains the large, well-supported (BP = 0.96) "laciniosa" group (units 19 – 25) in B2c, as well as *S. pascua* (unit 16) and their sister taxon *S. zelandica* (unit 26) in B2b, which shows a geographical differentiation (BP = 0.99) between samples from Japan and western Australia. The maximally-supported (BP = 1) sister taxon to both, B2a, is an unidentified species from Rottnest Island, western Australia, previously named *S. kurracheensis*, but clearly not that species.

Thus, with the solitary exception of *S. serrata* found in clade B, all South African species fell in clade A, and all but two of those in clade **β**.

Geographic distribution of clades

Several trends emerged relating to how phylogenetic groupings were reflected in biogeographic patterns of distribution (Fig. 4.3). The most obvious was that clade **β** dominates in Africa, being the exclusive occupant of the eastern Atlantic coast in both hemispheres and occurring along the whole of the east coast of the continent extending as far as the Persian Gulf. This clade was especially rich in southern Africa, with nine species. The only representative of clade **β** outside Africa was in the *S. pectinata* complex, represented by *S. naufragum* on the western Atlantic seaboard. Outside of Africa, members of clade A were absent from tropical regions apart from isolated island species and the above-mentioned presence of *S. naufragum* on the western seaboard of North America. On the subantarctic islands, clade A1 was a widely distributed and sole occupant.

With *S. thersites* on the northwest coast of North America and *S. fuegiensis* and *S. lateralis* at the southern tip of South America the members of clade A1 spanned the extremes of the Pacific west coast, with a conspicuous gap between them. *Siphonaria jeanae* and *S. diemenensis* (clade **i**) separated on their own in southern Australia, as did the south Australian and South American *S. funiculata, S. tasmanica* and *S. lessonii* in clade **α** and the New Zealand *S. australis* in clade A2c.

The almost circum-African clade **β** contains the Arabian *S. asghar* and all bar three of the South African species, with *S. capensis* replaced by *S. pectinata* north of Angola, and including its eastern north American counterpart, *S. naufragum*.

The Pacific clade A2b contains the Pacific *S. normalis* and *S. japonica* with the South African *S. compressa*, *Siphonaria* nov. sp. 3 from Knysna and one individual from Oman being geographically the odd men out.

Members of clade B are prevalent in tropical America, where a grade of species emerged as basal to clade B. They were even more geographically diverse in the eastern Indo-Pacific, spanning tropical, subtropical and temperate regions in a swathe from Japan to southern Australia, which clearly constituted a focal point of radiation, housing multiple clades: B1a, B1c, B2a, B2b and B2c – the latter extending across the eastern Pacific islands. Clade B1b, comprising only *S. serrata*, was the sole African intruder into clade B.

Clade B1c (containing the “atra” group) and clade B2c (containing the “laciniosa” group) formed the bulk of clade B and were nested within the Central American basal species (*S. gigas*, *S. brannani*, *S. maura*, *S. alternata*), which have nested within them the Indo-Arabian *S. savignyi*, *S. kurracheensis,* and the Red Sea species, *S. belcheri*.

Clade B1 is Indo-Pacific, spanning from the east coast of Africa to Japan in the north, through maritime southeast Asia and to the east coast of Australia in the south. The oddity is that the isolated South African *S. serrata* (clade B1b) fell within it. Clade B2 spanned the eastern Pacific from Japan across southeast Asia including Australia.

Apart from substantial overlap in Australasia, clades A and B therefore largely occupy different oceanic regions.

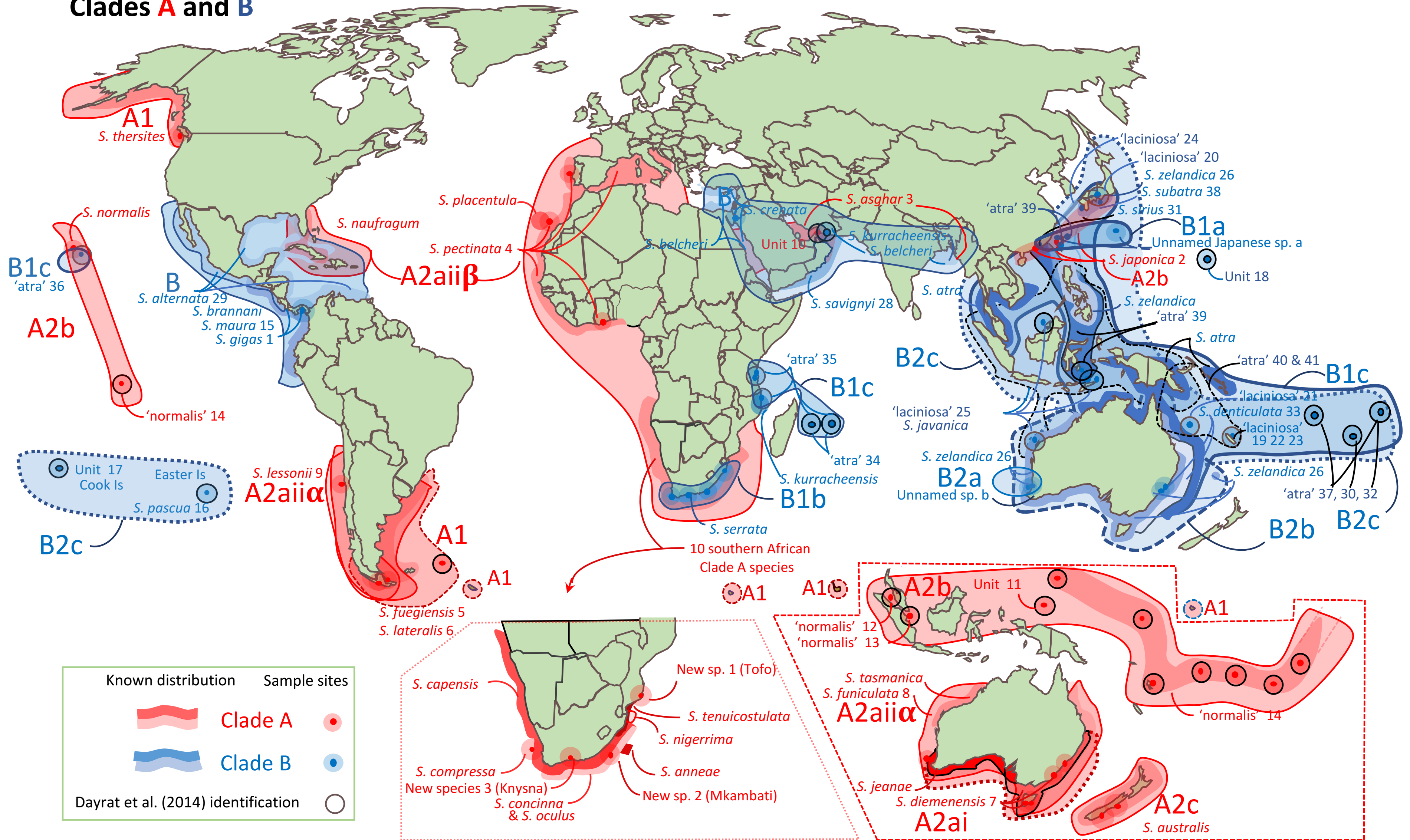

Figure 4.3 World map showing locations of all species and units sequenced for this thesis and their distributions. Inset bottom centre are details of the southern African species; members of clade A in Australasia have been offset bottom right to separate them from clade B members in the same region.

Latitudinal trends

There were strong latitudinal trends in the species richness of *Siphonaria.* None were found in the Arctic or Antarctic realms, but diversity then rose towards the lower latitudes before dipping at low latitudes in proximity to the equator (Fig 4.4). Departures from the null hypothesis were highly significant (χ^2 = 142.7, df = 15, P < 0.001).

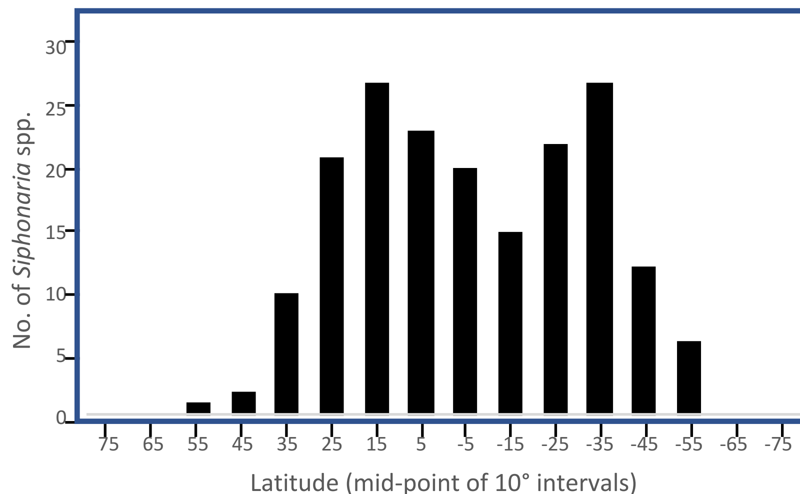

Figure 4.4 Trends in the species richness of *Siphonaria* species in relation to latitude, plotted for the midpoints of 10° intervals of latitude (data derived from Appendix 4.2 and references therein.

Geographic ranges relative to larval types

Several patterns emerged from my comparison of geographic ranges of species with different larval development types. In terms of latitudinal ranges (Fig. 4.5A), direct developers were almost all crowded together with small geographic ranges (with the exception of *S. kurracheensis*), whereas planktonic forms had both larger ranges and a greater variability in their ranges. Forms with 'swimming/crawling' larvae were scattered among range sizes and too few to draw any robust conclusions about them. Differences between species with planktonic vs. direct development were highly significant (one-tailed t-test, t = 2.28, df = 10, p = 0.0084).

Similar patterns emerged when maximal linear distances (rather than latitudinal extent) were considered (Fig. 4.5B), but there was one major departure in the form of an outlier: *S. lateralis* had a range three times that of any other species and, together with *S. kurracheensis* and *S. thersites*, were the only direct developers to fall within the spread of values for species with planktonic larvae. If *S. lateralis* was included in the analysis, there was no significant difference between direct and planktonic developers (one-tailed t-test, t

= 0.338, df = 11, p = 0.370). But if this outlier was excluded, the difference became highly significant (t = 4.61, df = 22, p = 0.000075).

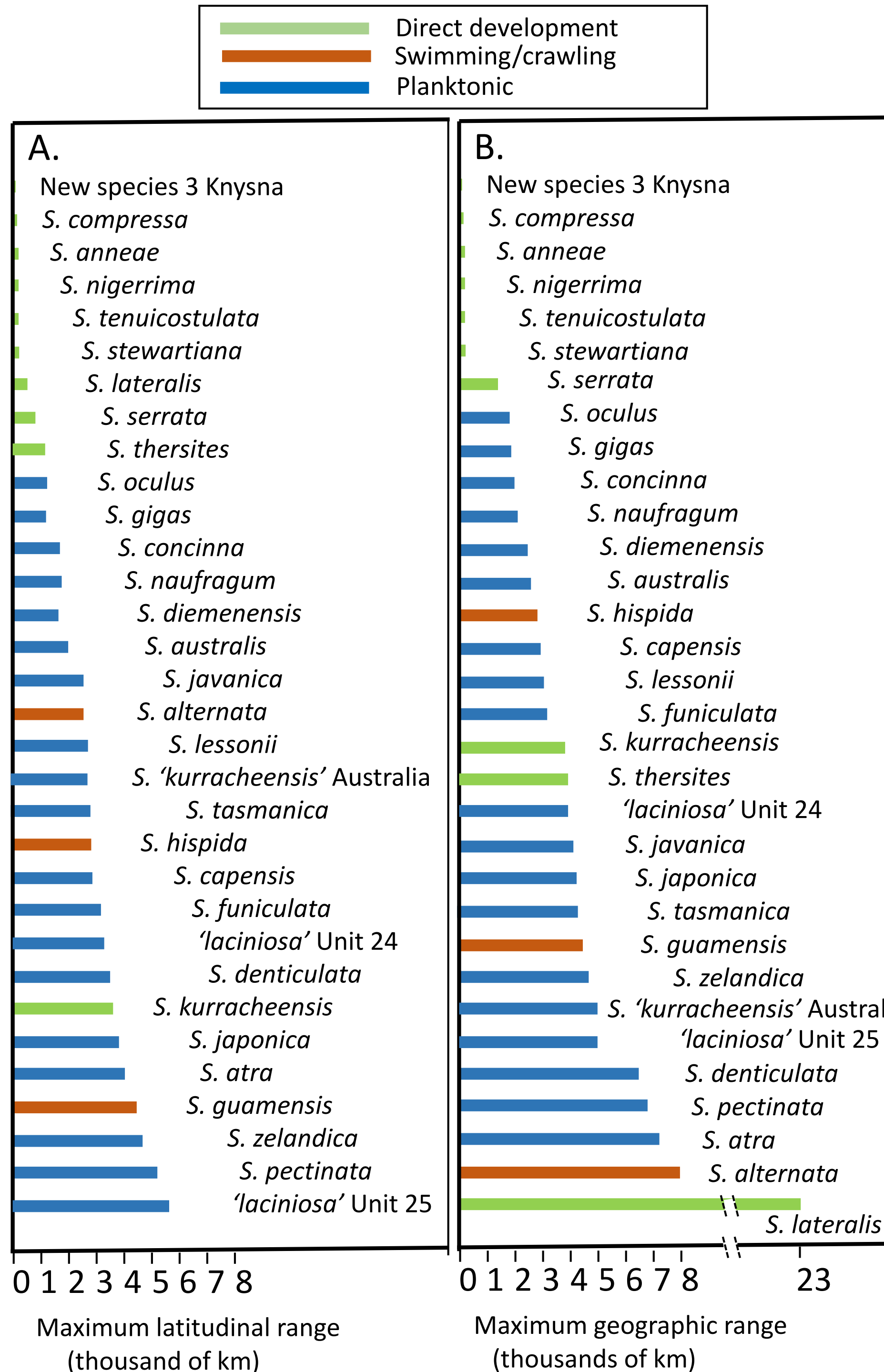

Figure 4.5 Ranges (thousands of km) for *Siphonaria* species: (A) greatest latitudinal range, and (B) maximal overall range, in relation to larval development type. Data for the first six species have been doubled to improve visibility. Data derived from Appendix 4.2 and references therein, and ranges portrayed in Figure 4.3.

The average latitudinal range per species was also greater for those with planktonic larvae (26.24°) than those with direct development (12.5°) (one-tailed t-test, $t = 4.17$, $df = 21$, $p = 0.00019$). Species in both categories were concentrated in the southern hemisphere, the mean centre of distribution for planktonic species being 11.2°S, and that for direct developers further south at 33.2°S.

The numbers of species employing planktonic larval development was high at low- to mid-latitudes, declining towards the poles (Fig. 4.6A), while the relative proportions of direct developers rose (Fig. 4.6B).

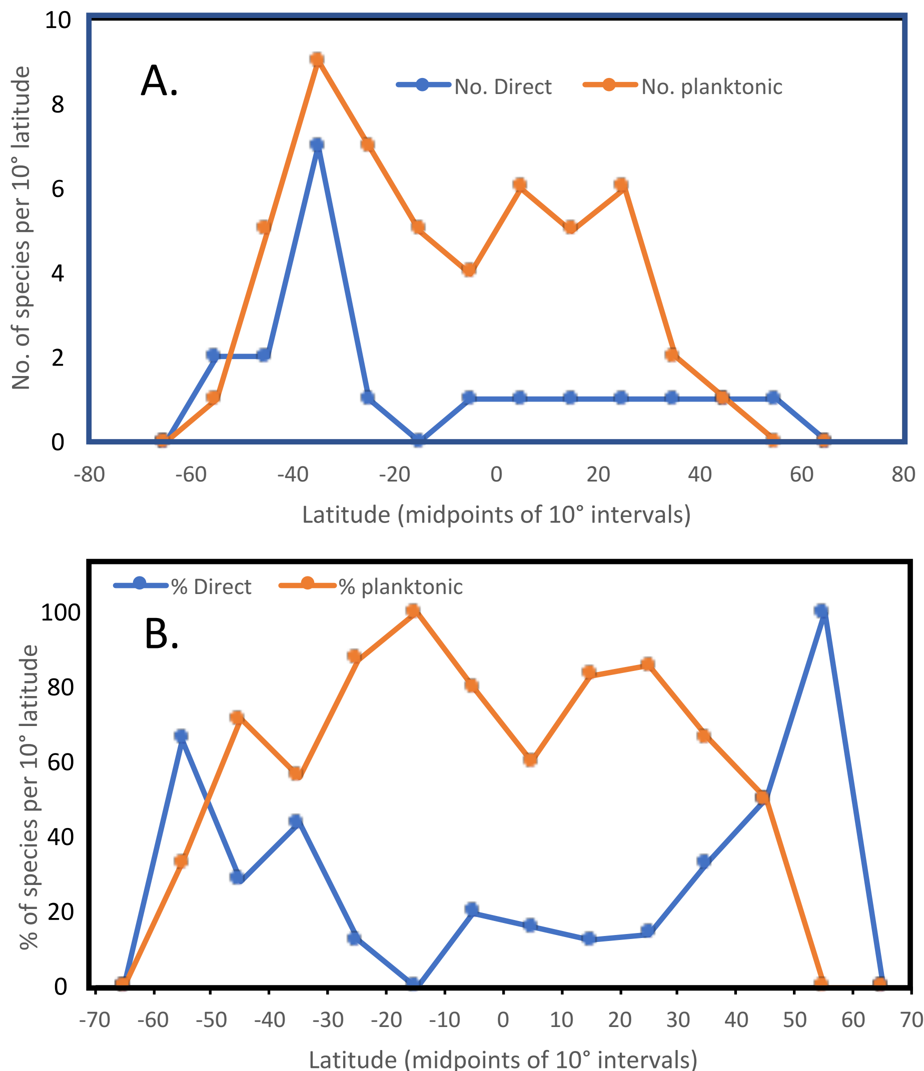

Figure 4.6 (A) numbers of species and (B) percentages of species that have planktonic or larval development, per 10° intervals of latitude. Negative figures are °S, positive values °N. Data derived from Table 4.2.

4.4 DISCUSSION

My discussion will focus on four areas. Firstly, I assess the similarities and differences between my phylogeny and that of the most recently published *Siphonaria* taxonomy (Dayrat *et al.* 2014). This will include addressing the placement of all the South African species, which are here for the first time represented in a global context. Secondly, I discuss the phylogeny in the context of qualitative shell features. Thirdly, I will compare my phylogeny with the subgeneric and 'sectia' divisions recognized by Hubendick (1946) to ascertain the extent to which they correspond. Finally, I will discuss the geographic distribution of clades that have emerged from my phylogeny.

Comparisons between my phylogeny and that of Dayrat et al. (2014)

The trees recovered in my analyses are congruent with those of the Dayrat *et al.* (2014) phylogeny in most cases. The monophyly of *Siphonaria* is upheld, and both analyses found two major clades within the genus. There are, however, also significant departures between my phylogeny and that of Dayrat *et al.* (2014), and the addition of the South African species adds an extra dimension. I will deal with these differences as they occur as one moves through the two major clades in Figures 4.2A and 4.2B. Unit numbers refer to the 41 units of named and un-named species described by Dayrat *et al.* 2014 (Appendix 4.1).

Clade A

Clade A diverged into two clades, A1 and A2. Clade A1 corresponds with the Dayrat *et al.* clade A units 5 and 6, and is a Western Pacific branch extending across the sub-Antarctic, containing the sister taxa *S. thersites* and an unnamed species from the Natural History Museum labelled *Kerguelenella lateralis* (the previous name of *S. lateralis*), recorded from Tierra del Fuego. I identified the "lateralis" unit 5 from Tierra del Fuego as *S. fuegiensis* from the original description of *S. fuegiensis* (Güller *et al.* 2016, González-Wevar *et al.* 2017) and the "lateralis" group unit 6 as *S. lateralis*. There were nine nominal species listed for the area by Güller *et al*. (2016) but only three have been confirmed (*S. lessonii*, *S. fuegiensis* and *S. lateralis*). For example, *S. macgillivrayi* has been assigned to *S. fuegiensis* (Güller *et al.* 2016). There are, however, other species that I could not examine genetically, that are likely to be members of this clade, based on their shared biogeography in the sub-Antarctic and their common shell form, notably *S. macquariensis* and *S. stewartiana*.

Clade **i** comprises the two Australian species *S. jeanae* and *S. diemenensis*. The analysis of Dayrat *et al.* (2014) did not include *S. jeanae*, and I can now identify their unnamed unit 7 as *S. diemenensis*. In clade **α**, *S. tasmanica* from Australia (not represented in the previous

phylogeny) is sister to *S. lessonii* (unit 9) from southern South America. *Siphonaria funiculata* (unit 8), also from Australia, is sister to both in that clade.

The genetic differentiation between Chilean and Argentinian samples of *S. lessonii* confirms earlier work that found two distinct lineages that were all but reciprocally monophyletic between the Pacific and Atlantic coasts of South America (Nuñez *et al.* 2015, Fernández Iriarte *et al.* 2020). The sequential arrangement of units 5-9 in my clades A1, **i** and **α** reflects close accordance between my phylogeny and that of Dayrat *et al.* (2014) for these units.

Clade **β** contains all the South African species bar three: *S. serrata* found in clade B, *S. compressa* and its sister, the new species 3 from the Knysna Estuary in South Africa (see Chapter 3), both found in clade A2b. Thus, the South African taxa do not form a monophyletic radiation. However, setting aside the three exceptions, all the remaining 9 South African species were found in this single clade; moreover, with the exception of *S. serrata* found in clade B, they all fall in clade A.

This global phylogeny confirms the results of Chapter 2 with the additional resolution of the polytomy between *Siphonaria* nov. sp. 1 from Tofo (Mozambique), *S. tenuicostulata* (Chidenguele, Mozambique) and *S. nigerrima*. *Siphonaria nigerrima* and the new species 1 from Tofo were recovered as sister species, as were the nov. sp. 2 from Mkambati and *S. anneae* while *S. tenuicostulata* is positioned as sister taxon to this pair.

Siphonaria asghar (unit 3), endemic to the Persian Gulf and the Gulf of Oman (Dayrat *et al*. 2014), is sister taxon to all five east coast South African and Mozambican species in the 'nigerrima' complex dealt with in Chapter 2. *Siphonaria concinna* and *S. oculus* are the sister taxa to this clade. This is an additional confirmation of the results in Chapter 2 that show *S. oculus* and *S. concinna* are distinct species and sister taxa. Within this clade of closely related species, support varies from 1.0 to 0.6. If these sister taxa are a result of recent emergence or incipient speciation then lineage sorting and hybridization may still occur and this would reduce apparent reciprocal monophyly and explain the lower clade probabilities (Shaffer and Thomson 2007).

Displacement of *S. asghar* (unit 3) and *S. pectinata* (unit 4) from their original sequence in the phylogeny of Dayrat *et al.* (2014) phylogeny is merely a reflection of the fact that they included no southern African species, the majority of which were united with *S. pectinata* and *S. asghar* in clade **β**. This clade in all likelihood extends to cover the whole of the African coast, as indicated by the inclusion of *S. tenuicostulata* in the Western Indian Ocean fauna (Richmond and Rebesandratana 2010).

Siphonaria capensis is clearly closely related to *S. pectinata* (unit 4) to which it gives way at some point between northern Angola and Ghana on the west coast of Africa. *S. pectinata* (sensu lato) displays strong differentiation between the American and European/African individuals, as Kawauchi and Giribet (2011) have documented. These lineages have now been recognized as a separate species, *S. naufragum*, occurring in Florida and the Gulf of Mexico (and possibly extending to Venezuela), whereas *S. pectinata* occurs in the eastern

Atlantic and the Canary Isles (but not Madeira or the Azores) and Mediterranean (Giribet and Kawauchi 2016). Moreover, a third species, *S. placentula*, has been genetically identified from the Cape Verde Archipelago and is closely allied to *S. pectinata* (Giribet and Kawauchi 2016), making it reasonable to assume it is part of this clade. Espinosa *et al.* (2016) argue for recognition of the two transatlantic populations as a single species, *S. pectinata*, but their data reveal a clear-cut genetic distinction like that used by Giribet and Kawauchi to advocate separation of the species, and I favour the latter view.

Early views that '*S. pectinata*' on the eastern seaboard of America might have been a human-introduced alien from Africa (Morrison 1963) have been countered by Vermeij and Rosenberg (1993) who argue for a natural transoceanic transfer (along with at least 14 other molluscan species) predating human influence. Genetic differences and recognition that the two transatlantic populations constitute different species reinforce the antiquity of the differences (Kawauchi and Giribet 2011, Giribet and Kawauchi 2016). *Siphonaria pectinata* (sensu stricto) has, however invaded the Mediterranean, progressively spreading eastwards in recent years (Gofas and Zenetos 2003, Crocetta 2016, Slama *et al.* 2018).

Bosch *et al*. (1995) and Dayrat *et al.* (2014) report four units from Oman – *S. asghar* (unit 3), an unnamed unit 10, *S. kurracheensis* (unit 27) and *S. savignyi* (unit 28). The unnamed unit 10 is one individual from the Arabian Sea at Oman, and is described as being similar to *S. compressa*, having the inner shell white to light brown with white rays that are sometimes absent and which extend beyond the muscle scar. Unit 10 is the very well supported sister taxon to the South African *S. compressa* and the new species 3 from Knysna(described in Chapter 3), and is thus of particular interest. It shares minute size with these species and is also described as occurring in a lagoon – among weed and rocks (Bosch *et al.* 1995). *S. asghar* and the unnamed unit 10 occur in clade A (as does *S. compressa* and new species 3) while the other two, *S. kurracheensis* (unit 27) and *S. savignyi* (unit 28), as well as the Red Sea *S. belcheri*, were in clade B.

In clade A2b, my samples from Oahu, Hawaii, identified by C. Bird (pers. comm.) as *S. normalis*, were resolved as the sister group to a sample from the Caroline Islands (unit 11), which together form the sister taxon to the "normalis" group units 12, 13 and 14. Dayrat *et al.* (2014, p 267) state that "Only unit 14 can be called *S. normalis*"; but my specimens from Hawaii emerge separately from Units 12-14. Hawaii is the type locality for the species, so I am confident in allocating the species name to that locality, as did Hubendick (1946), who regarded the species as being endemic to Hawaii. Whether my samples or those of unit 14 should be considered to be this species is ambivalent, as some of the specimens incorporated in unit 14 also came from Hawaii. More research is required to resolve this issue.

Clade A2c comprised only *S. australis*. This species was not included in the analysis of Dayrat *et al.* (2014), although they do comment (p 266) that their unit 7 (now identified as *S. diemenensis* on grounds of shared shell structure and inclusion of an identified specimen), "shares some similarity with *S denticulata* … *S. australis* and *S. diemenensis*".

However, my analysis clearly separates all three species, with *S. australis* forming clade A2c, *S. diemenensis* falling in subclade A2ai, and *S. denticulata* being so different that it falls in clade B1c.

In this context it is worth noting that the Atlas of Living Australia (https://bie.ala.org.au, accessed 15-12-2020) lists *S. denticulata* as a synonym of *S. diemenensis*, based on Hubendick's (1946) view that that they are synonymous. This is clearly not correct, for although their shells are similar, they are genetically so different that they fall respectively in clades B and A. The website Sea Shells of New South Wales (2020) has the two as both being valid species, as does MolluscaBase (2020).

Clade B

The initial splitting of clade B was via progressive divergence of single taxa. *Siphonaria gigas* (unit 1), was the basally divergent species and it formed a graded series together with *S. brannani*, *S. maura* (unit 15), *S. alternata* (unit 29), *S. savignyi* (unit 28), *S. kurracheensis* (unit 27) and *S. belcheri* (from the Red Sea). *S. maura* (unit 15) is shown as including UF141401 in Figure 4.2B; this placement differs somewhat to where this sequence is in the Dayrat *et al.* (2014) phylogeny. In my phylogeny it occurs as a well-supported split (BP = 0.80) from the rest of the *S. maura* sequences.

S. gigas formed a basal divergence from the rest of clade A in Dayrat *et al*. (2014). In some of my preliminary exploratory trees (not shown), it formed a deeper split, sister to both A and B, sometimes together with *S. maura.* Its precise position relative to other species therefore needs further resolution; but it is clear that it stands as a deep divergence within the genus.

Siphonaria brannani was not included in the Dayrat *et al.* (2014) phylogeny and *S. maura* appeared as basal to their clade B (as it does in my clade B). However, their *Siphonaria alternata* individuals from Florida occupied a very different position in their phylogeny from mine, allied to three species with widely different geographic positions: *S. zelandica* from Australia, and *S. kurracheensis* and *S. savignyi* from Oman. The fact that *S. gigas*, *S. maura, S. brannani* and *S. alternata* all occur in the same area on the eastern Pacific coast of Panama and adjacent areas tends to support their position as a graded series in clade B in my phylogeny.

Three species that I added to the phylogeny – which were not represented in the Dayrat *et al.* (2014) analysis – emerged near the base of clade B. Specimens from Eilat in the Red Sea identified as *S. belcheri* on the basis of photographic similarity with specimens shown in two sources (Bosch *et al*. 1995, Albayrak and Çağlar 2006), were retrieved as the sister taxon to the bulk of clade B. The second species, which fell in B1a, is an unnamed species from Japan, and was sister to clades B1b and B1c. The third addition was *S. serrata* (B1b), which is a surprise, being the only southern African species in the otherwise strongly Indo-Pacific clade B. *Siphonaria serrata* was sister to the large clade B1c, which comprised the

‘atra’ group (Units 30-41) including *S. sirius, S. subatra* and *S. denticulata*. The ‘atra’ group spans a huge geographic area, including Japan, Tonga, Samoa (units 30-32 and 38); the south and east coasts of Australia (units 33-34); Tanzania, Mozambique, Mauritius, Reunion and a (seemingly unlikely) record for Thailand (unit 35); Hawaii (unit 36), Samoa and Fiji (unit 37), Philippines, Taiwan, Sulawesi (unit 39) Papua New Guinea; New Caledonia and Queensland (units 40-41). The unification and sequence of units 30-34 in my phylogeny repeats almost exactly those evident in the Dayrat *et al.* (2014) phylogeny.

Confusion exists around the names *S. crenata* (which does not feature in my phylogeny), *S. belcheri*, *S. savignyi* and *S. kurracheensis.* According to Zenetos *et al.* 2010, the origins of *S. crenata* are in the Persian Gulf and the Arabian Sea, and it extends to the Red Sea and the Suez Canal. Its occurrence in the eastern Mediterranean, specifically SE Turkey and Israel (Galil *et al.* 2020), and that of *S. belcheri*, constitute alien invasions (Delongueville and Scaillet, 2010). *Siphonaria belcheri* is purportedly also from the Arabian Sea and Persian Gulf and occurs in the Mediterranean but not in the Red Sea or Suez Canal (Dekker and Orlin 2000, Albayrak and Çağlar 2006). *Siphonaria savignyi* is said to be distributed from the Gulf of Oman to the Red Sea (Dayrat *et al.* 2014). Further complicating the issue are photographs from the Natural History Museum Rotterdam of two completely different looking shells, both identified as *S. crenata*. The one (WoRMS 68682/NMR 64714) is similar to the photographs of *S. crenata* in Delongueville and Scaillet (2010), while the other from the Gulf of Aqaba, an extension of the Red Sea (WoRMS 68683/NMR34960), resembles the *S. belcheri* in Bosch *et al*. (1995) and Albayrak and Çağlar (2006). And finally, syntypes of *S. belcheri* from the Natural History Museum UK look like neither of the above photographs.

In my phylogeny, the ‘Red Sea species’ I have named *S. belcheri* is a well-supported terminal taxon genetically distinct from *S. savignyi* (unit 28) and from *S. kurracheensis* (unit 27), all three of which form successively sister monophyletic groups.

I resolved to base my identification of my Red Sea individuals on the excellent photographs of what were identified as the Mediterranean invasive *S. crenata* occurring in Iskenderun Bay, Turkey (Delongeville & Scaillet 2010) and *S. belcheri* (Albayrak & Caglar 2006). My shells are distinctly different from those of *S. crenata* shown in Delongueville and Scaillet (2010) and comparable to the *S. belcheri* images in Bosch *et al*. 1995 and Albayrak and Çağlar 2006.

The placement of *S. kurracheensis* from Oman by Dayrat *et al.* (2014) as unit 27 is of interest as the two individuals from Oman they identified as this species linked strongly together with an unidentified Natural History Museum sample from Pemba Bay, Mozambique that I included in my analysis. Dayrat *et al.* (2014) suggest that the distribution of *S. kurracheensis* is restricted to the Arabian Sea, the Persian Gulf and the Gulf of Oman, with the type locality in Karachi, Pakistan (Reeve, 1856), and that it is not as widely distributed as originally proposed by Hubendick (1946). This is problematic as the Mozambican sample is phylogenetically well supported and either falls within the

definition of *S. kurracheensis*, or is a closely related sister species. It was identified and confirmed as being *S. kurracheensis* by a GenBank *S. kurracheensis* sequence (KM492939 from Sindh, Pakistan) obtained after my phylogeny was completed.

Further complicating the picture are samples I received from Rottnest Island, western Australia, which were identified as *S. kurracheensis* by the collector. These emerged as the sister taxon to *S. zelandica* and the "laciniosa" group in clade B2. This '*S. kurracheensis*' is described as being distributed along Western Australia (Black and Johnson 1981, Johnson *et al.* 2001), with isolated records from Victoria and Queensland (Atlas of Living Australia 2020). It is clear that this '*S. kurracheensis*' is not genetically related to the species described from the Arabian Sea and I have termed it "unnamed species b Rottnest Island".

In short, *S. savignyi*, *S. kurracheensis* (unit 27 and my specimen from Mozambique) and *S. belcheri* from the Red Sea all emerge as genetically related as part of a grade but distinct in my phylogeny, and warrant specific recognition. However, the species currently bearing the name '*S. kurracheensis*' that is considered to be widespread in Australia (Atlas of Living Australia 2020) is unrelated to the true *S. kurracheensis* and probably represents an undescribed species.

Clade B1c contains the "atra" group (units 30–41) within which are found *S. sirius* (unit 31), unit 33 and unit 38 (*S. subatra*). There is no clarity on which of units 30-41 should be assigned to the name *S. atra*. I consider that unit 33 is *S. denticulata* because of (a) the inclusion of a GenBank sequence bearing this name in the unit; (b) coincidence of the geographic range of unit 33 with that of *S. denticulata*; and (c) similarity of shells.

Siphonaria atra, *S. guamensis* and *S. javanica* are found in Singapore and the South China Sea between Malaysia and Borneo (Tan and Kastoro 2004, Chim and Tan, 2009). As both unit 13 (falling within the "normalis" group in clade A) and one of the numerous individuals comprising unit 25 (UF292268) within the "laciniosa" group were recorded from Singapore by Dayrat *et al.* 2014, I speculate the identity of units 13 and that specific individual of unit 25 may be two of the species. If *S. atra* is presumably part of the "atra" group (one of the unnamed units 32, 30, 34 - 37, 39 - 41) then units 13 and 25 may be *S. guamensis* and *S. javanica* respectively.

My sample Indns377BBBM from The Natural History Museum from Sulawesi, Indonesia, falls within unit 39, and was labelled "*Siphonaria* cf. *javanica*" by the collector, M. Malaquias. This would suggest either unit 25 or unit 39 may be *S. javanica*, placing the taxon well within clade B. A sequence of *S. javanica* (MN690503) obtained after my analysis was complete and thus not included in my final tree shows strong similarity to my sequence from East Timor which appears in unit 25. Thus, I tentatively suggest, based purely on this limited sequencing evidence, that unit 25 is in fact *S. javanica*, which would mean that unit 13 is possibly *S. guamensis*.

The photographs of the individuals I sequenced that fell into unit 25 compared favourably with photographs of *S. javanica* identified by Ria Tan

(http://www.wildsingapore.com and https://singapore.biodiversity.online/species/A-Moll-Gastropoda-000563).

Two species from Hong Kong are difficult to tell apart: *S. sirius* and *S. atra*; and Slingsby *et al.* (2000) have proposed that they may be ecomorphs of *S. laciniosa*. This poses a problem as *S. sirius* falls within clade B1 in the "atra" group whereas the "laciniosa" group falls some distance from it, within clade B2. However, the addition of six sequenced *S. sirius* individuals from Hong Kong and Japan provides strong support for recognition of this species. As *S. japonica* – the other well-established species in Japan, Hong Kong and Taiwan – is well supported in clade A, this leaves *S. atra* as a possible name for the unknown in Hong Kong. Given that Dayrat *et al*. (2014) designated a large "atra" group containing individuals from Papua New Guinea, New Caledonia and Samoa, which form a triangle neatly encompassing Vanikoro in the Solomon Islands from where *S. atra* was first described, *S. atra* might be one of the following unnamed units in the "atra" group: 32, 30, 34 - 37, 39 - 41. Unfortunately Dayrat *et al.* did not include any individuals from Vanikoro in their analysis, making it difficult to decide which unit should inherit the name *S. atra*.

Clade B2b comprises only *S. zelandica*; and its recognition as a discrete unit is strengthened by my addition of six additional sequences.

Clade B2c contains the large "laciniosa" group (units 19 – 25, as well as *S. pascua* (unit 16) and units 17 and 18; and its sister taxon is *S. zelandica* (unit 26). Additional sequences added to these units confirm they are robust discrete units in both my phylogeny and that of Dayrat *et al*. (2014).

A major difference between my phylogeny and that of Dayrat *et al.* (2014) is that whereas they found that *S. gigas* (unit 1) and *S. maura* (unit 15) were at the bases of clades A and B respectively, my result showed that *S. thersites* (from the Pacific north-west America), and *S. lateralis*, *S. fuegiensis* and an unnamed species related to *S. thersites* (from the Pacific south western America and Kerguelen Island) were at the base of clade A; while for clade B there is a set of successive species, the three species lying closest to the base, *S. gigas*, *S. brannani* and *S. maura*, being distributed along the north American tropical Pacific west, with a fourth, *S. alternata,* additionally recorded from Florida and Bermuda (Cook 1971). Within the rest of this grade, there are three Afro-Indian species, *S. savignyi*, *S. kurracheensis,* and *S. belcheri*, which are basal to the bulk of the Indo-Pacific species including all members of the "atra" and "laciniosa" groups.

Common south-eastern Australian species include *S. tasmanica*, *S. funiculata*, *S. diemenensis* (all in clade A), *S. denticulata* and *S. zelandica* (both in clade B) (Colgan and da Costa 2013). Thus, the Australian taxa are also not monophyletic.

Correlates between phylogeny and shell form

The focus of my study was on genetic resolution of the affinities among *Siphonaria* species, not on shell morphometrics. Nevertheless, it is worth noting that clear groupings of shell types coincided with some of the clades I recognised (see Figure 4.2A, B).

The first was in a set of four related species in clade A1, sister to the remainder of clade A: *S. thersites*, *S. lateralis*, *S. fuegiensis* and as-yet unnamed material from Kerguelen Island. All had fragile, relatively flat, shells of moderate size (10-15 mm) that were strongly asymmetric, with the apex positioned far back and approaching the left margin of the shell.

The second lay more deeply embedded in clade A, in clade A2b: *S. compressa* (and its sister, the new species 3 from Knysna, dealt with in Chapter 3), as well as their closest relatives, unit 10 from Oman, and unit 11 from Caroline Island. They too had asymmetric 'hooked' shells with markedly posterior apices in three cases; but the most obvious connection is their small size: respectively around 6, 2, 4 and 4 mm. Small size is, of course not necessarily a good indicator of taxonomic relationships. The miniaturisation of *S. compressa* and its sister taxon, reflects their habitat on the narrow blades of eelgrass, *Zostera capensis* (Angel *et al.* 2006, Allanson and Herbert 2005). The only comparably small species in the Siphonariidae are *Aporemodon tomlini* at 2.9 mm (Robson, 1913) and *Siphonacmea oblongota* at 7 mm (Toyohara *et al.* 2001). The latter is of special interest as it too lives on blades of *Zostera* spp., implying convergent evolution of this species with *S. compressa*.

The remainder of the clade A species are very different from the above species, being almost oval, with an approximately central apex, minimal projections of the costae at the margin, and with the siphonal canal projecting only slightly. The members of the 'African' clade **β** are particularly similar.

Members of clade B are generally strikingly different from those in clade A, being more robust with fewer primary costae that, in many cases, project markedly to create a star-shaped pattern and a strongly crenulate margin. *Siphonaria serrata*, the sole African representative, stands out as being more like the shells of clade A species, although it does have distinctive prickles that project from the ribs, especially during the young phase. *Siphonaria gigas* is the giant of the group, reaching 60-80 mm, challenged only by the 50-mm fossil species *Siphonaria vasconiensis* (Harzhauser *et al.* 2017).

In short, although I did not analyse the morphometrics of shells, there were some species in parts of the phylogeny that have similar shell morphology and clear correlations with the phylogeny retrieved, despite the multiple and confusing overlaps in shell morphology among species that compelled Dayrat *et al.* (2014) to recognise genetic units that were indistinguishable morphologically.

Consideration of genera, subgenera and 'sectia'

My phylogeny and that of Dayrat *et al.* (2014) resolve the species of *Siphonaria* dealt with (in the broader sense) into two major clades, which could justifiably be elevated to the stature of genera. There have, however, been a number of other attempts to divide the group or create genera, and they need to be considered in relation to the emergent phylogeny.

The most important of these is that of Hubendick (1946), who split the genus into two subgenera, *Siphonaria* and *Liriola*, and divided those among several 'sectia'. Many authors have treated the latter as subgenera (e.g., Christiaens 1977, Chambers and McQuaid 1994a, b). Fig. 4.7 distils my phylogeny and overlays the subgenera and sectia of Hubendick. Subgenus *Liriola* comprises predominantly members of clade A apart from three units in clade B; but subgenus *Siphonaria* pools various representatives of both clades A and B. This supports the view of Dayrat *et al.* (2014) that neither of Hubendick's subgenera are natural monophyletic entities. I do not wish to assign names but, should a split be made, I suggest that since the type of *Siphonaria* falls within Clade B that this would be *Siphonaria* sensu stricto and the oldest name for Clade A would be *Liriola* Dall 1870.

Turning to the 'sectia' of Hubendick (1946), some do constitute strong natural groupings. Within the subgenus *Liriola*, the sectia *Liriola* and *Kerguelenia* (now named *Kerguelenella*) constitute a collective unit with a common shell form (see above). They emerge as monophyletic, incorporating *S. thersites*, *S. lateralis*, *S. fuegiensis* and particularly interesting unnamed specimens from Kerguelen Island that are – despite their considerable geographic separation – sister to *S. thersites*, which occurs on the eastern Pacific shores of North America. To that group can probably be added *S. innomonata, S. tristensis*, *S. stewartiana*, *S. macgillivrayi* and *S. macquariensis*, judging from their shared shell form and co-occurrence with other members of the sectio *Kerguelenella* on the sub-Antarctic Islands. Indeed, *Kerguelenella macquariensis* is currently recognised as falling in the genus

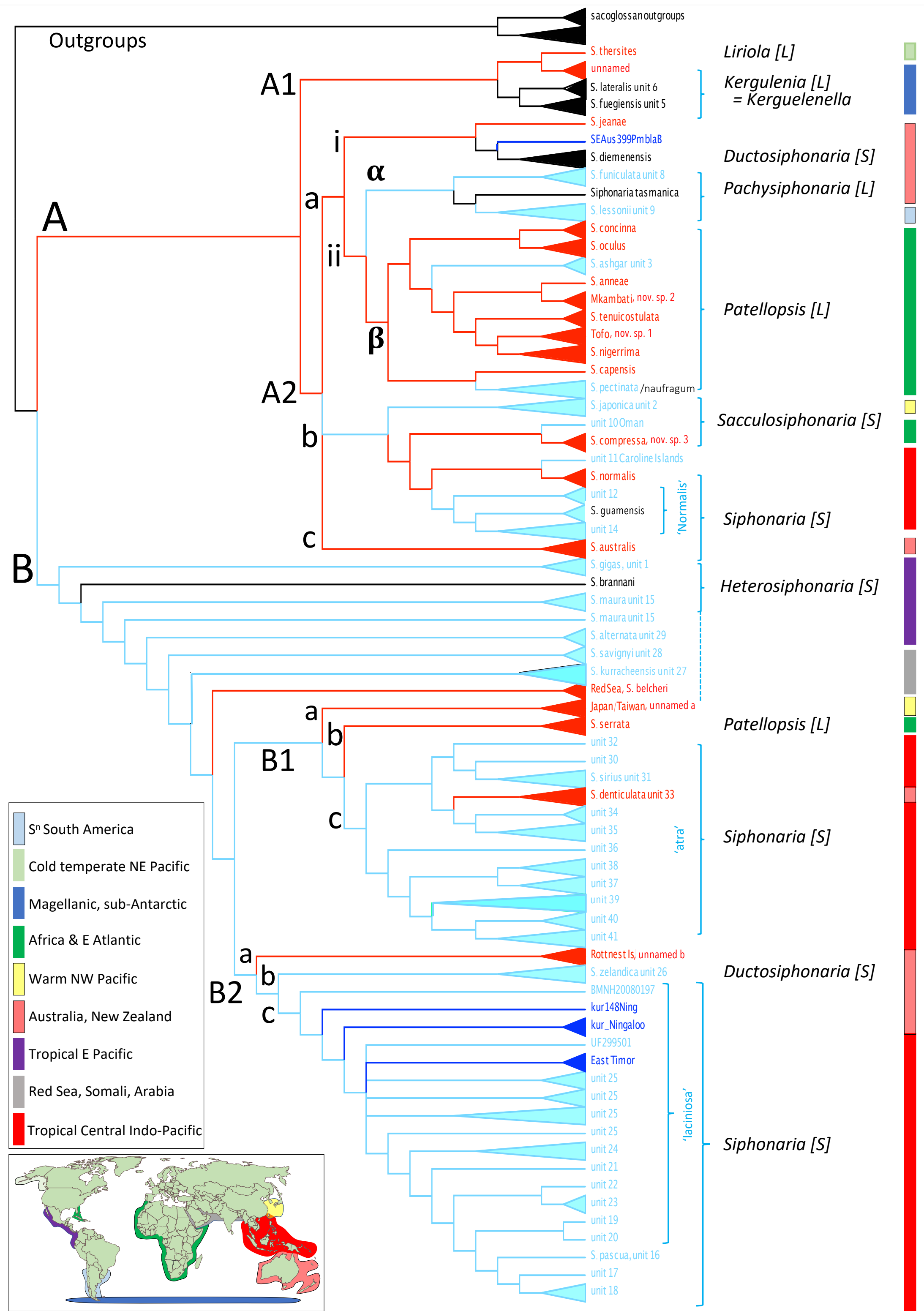

Figure 4.7 Simplified topology based on the phylogeny in Figure 4.2, indicating relationships with Hubendick's (1946) sectia within the subgenera *Liriola* [L] and *Siphonaria* [S], and the geographic distribution of the clades in my phylogeny. The dotted line indicates species that might fall in sectio *Heterosiphonaria*.

Kerguelenella by MolluscaBase, and many of the other species listed above are allocated the name as a subgenus. The case for recognising the taxon at generic level is strengthened by the fact that it sits at the base of clade A and, with the exception of *S. thersites*, occupy a common geographic region.

A second distinctive group in the subgenus *Liriola* is *Patellopsis*, which in my phylogeny constitutes clade **β** and contains the majority of the African species, extending to the Persian Gulf and incorporating *S. naufragum* on the western Atlantic coast of North America, which constitutes a trans-Atlantic migrant and is a naturally-arrived offshoot of the West Africa species *S. pectinata* (Vermeij and Rosenberg 1993, Kawauchi *et al.* 2011). The only clear departure from Hubendick's *Patellopsis* is *Siphonaria serrata*, which was placed in my clade B.

Sectio *Pachysiphonaria* also constitutes a discrete genetic unit, corresponding to clade **α**, although I could not locate genetic analyses for some of the species included by Hubendick to test the validity of their inclusion.

Turning to subgenus *Siphonaria,* sectio *Heterosiphonaria* loosely corresponds to the series of basal species that emerged in clade B; but the equivalence is weakened by the fact that Hubendick included *S. cochleariformis* (since synonymised as *S. japonica*) whereas my phylogeny positioned that species in clade A2b. Confusion also reigns in Hubendick's placement of this species because, under the name *S. japonica*, he places it in sectio *Sacculosiphonaria*.

Sectio *Ductosiphonaria* is not a natural entity, as *S. baconi* (now synonymised with *S. zelandica*) falls in clade B2b, and *S. diemenensis* in clade **i**, so these two species in the section are distant, and the third species, *S. bifurcata*, has never been analysed genetically. To compound matters, *S. elegans* (also synonymised under *S. zelandica* and thus considered the same species) is placed in a different section, i.e., *Siphonaria*.

In short, based on these mitochondrial data, while two of Hubendick's sectia, *Patellopsis* and *Kerguelenella* can readily be defined (or redefined) to incorporate monophyletic units, the remaining sectia would require substantial revision and rearrangement of species in them, while still taking into consideration the position of the type species, for them to constitute valid phylogenetic units. It may be that a future multilocus analysis including more individuals and species and other loci would recover some of the sectia not currently supported.

I turn now to Iredale (1914, 1940), who was prolific in describing and naming siphonariids, mainly from the Australasian region, and allocated species to a plethora of genera, namely *Parellsiphon, Legosiphon, Planisiphon, Mallorisiphon, Mestosiphon, Simplisiphonaria*, *Ellsiphon, Triellsiphon*, *Talisophon* and *Torquisiphon*. Of the 24 species he described, 14 have been synonymised with other species in the genus *Siphonaria* or classed as 'taxon inquirendum'; five were recognised as valid but transferred to the genus *Siphonaria* by Hubendick (1946) and accepted as such by MolluscaBase; and only five are retained by MolluscaBase in the genera in which Iredale placed them: *Ellsiphon marza*,

Hebesiphon monticulus, *Mallorisiphon oppositus*, *Mestosiphon lentulus* and *Planisiphon soranus.* Hubendick (1946) raises doubts about the validity of all these species bar *H. monticulus*, and even that he places in the genus *Siphonaria*, subgenus *Siphonaria.* Placement of species in Iredale's genera depended solely on shell shape as he did not have the benefit of modern genetic approaches that are now available, and shell shape in the family is known to remarkably fickle as a means of separating species, let alone genera, with high plasticity in response to local factors such as shore height and wave action (Cooke 1911, Tablado and López Gappa 2001, Soria *et al.* 2017, Livore *et al.*, 2018). As Simone and Seabra (2017, p 148) note: "Forms of different shell structure have proved to be conspecific variations, while conchologically similar forms have turned out to be different endemic species". In summary, there seems no case for retaining the genera that Iredale (1914) erected to accommodate species that, almost without exception, can be incorporated in the genus *Siphonaria.*

One final generic placement warrants comment. The website LifeMap (2020), based on genetic affinities, isolates *Siphonaria sirius* as being sufficiently distinct to warrant placement in a separate genus, *Anthosiphonaria* (Kuroda *et al*. 1971). My placement of this species in clade B1 raises doubts about the advisability of erecting a separate genus for it, or even treating it as a subgenus (Kurozumi, 2000). However, given that *S. sirius* is the type species of *Anthosiphonaria*, it may serve as the supra-specific name for the "atra" group.

As with all naming decisions based on genetic differences – including my own – there is the possibility that genetically identified units have been misnamed in data bases.

The judicious approach would be to retain *Siphonaria* as the umbrella genus that accommodates the majority of the species within the family apart from those rigorously defined as belonging in separate genera, notably *Aporemodon*, *Siphonacmea*, *Pugillaria*, *Benhamina* and *Williamia*. There are four reasons for adopting this approach. First, the status of many species needs resolution. Second, until at least the majority of the species have been genetically analysed, precipitous placement of species is likely to lead to premature recognition of genera and subgenera and ignorance about where un-analysed species should be positioned within them. Third, given the multiple clades and subclades that exist, to establish alternative generic names that are consistent in terms of monophyly and exclude paraphyletic combinations would lead to a multiplicity of names. Finally, unless clear distinctions in shell form can be established among genera and subgenera, extinct, fossil species will always be difficult to place, whereas their inclusion in the genus *Siphonaria* is straightforward. As indicated above, there are hopeful distinctions in shell form at least between clades A and B and, more specifically, for clade A1 (*Kerguelenella*), which point the way to possible future divisions of the genus into subgenera.

Biogeographic patterns

There were no obvious correlations between the distribution of *Siphonaria* species or clades and the biogeographic realms distinguished by either Spalding *et al.* (2007) or Costello *et al.* (2017), but that is not surprising considering the relatively fine divisions among their realms compared to the geographic ranges of clades. There were, nevertheless, a number of geographic patterns that emerged in the distribution of *Siphonaria* species and their clades.

Drawing on Table 1.1 and my phylogeny, the genetic split into two major clades A and B was reflected in a biogeographic division, with clade A largely African and temperate to sub-Antarctic, whereas clade B was predominantly tropical, especially in the Eastern Indo-Pacific and Central America, although there were overlaps between the two (Figures 4.3, 4.7).

The Tropical Central Indo-Pacific emerged as a centre of exceptional diversification of the genus, as Dayrat *et al.* (2014) have noted previously: no less than 25 species (including unnamed genetic units) occur there, predominantly members of clade B.

The richness of biodiversity in this region is well known (Bowen *et al.* 2013, Huang *et al.* 2017), and has been amply demonstrated for several taxa, including bivalves (Shumm *et al.* 2019), corals (Veron *et al.* 2015), fish (Allan 2007, Parravicini *et al.* 2013) and even foraminiferans (Förderer *et al.* 2018). One possible reason for the radiation of *Siphonaria* species there is that the Coral Triangle in particular, but also the Central Indo-Pacific region as a whole, comprises multiple islands and island archipelagos, offering opportunities for isolation, speciation and exchange among islands.

In this context, the diversity of *Siphonaria* species offers some interesting perspectives. Within the Tropical Central Indo-Pacific, islands that can be regarded as 'coastal', such as Sumatra, Borneo, New Guinea, the Philippines and Indonesia, harbour a larger number of species (13) than those to the east that are 'offshore' (10). Although the difference is not large, the pattern does conform to the predictions of Vermeij (1972) that extreme isolation will decrease the number of species. Indeed, Vermeij (1987) has gone further to suggest that very isolated single islands should be depauperate because of the difficulties larvae may have in reaching them. This does seem to be the case for *Siphonaria* species: Ascension and St Helena islands lack any *Siphonaria* species (Vermeij, 1972, Brown *et al.* 2016), and Easter, Cook, Wake and the Marshall Islands each have single species (Dayrat *et al.* 2014; and see Table 1.1). Dayrat *et al.* (2014) also point out that many of their unnamed units (10-13, 16-19, 30, 32, 36 and 38) are endemic to small areas, particularly islands – although New Caledonia, a continental island, harbours at least three units. Set against the argument that substantial isolation is associated with few species because of limitations on dispersal, the sub-Antarctic chain of widely spaced islands, while conforming in harbouring a small diversity, contains species such as *S. lateralis* and *S. fuegiensis* that are found on all or most of the islands. Expressing surprise at this, González *et al.* (2018) title their paper

reporting this as "Unexpected absence of island endemics". They infer that long-distance dispersal, possibly associated with drifting plants of the bull kelp *Durvillaea antarctica*, accounts for the wide geographic spread of the species. This island chain is, additionally, unusual in that it lies in the Antarctic Circumpolar Current, the only current to circumnavigate the globe uninterrupted by continents, which may aid dispersal among the islands. Johannesson (1988) has also noted that if species with direct development do manage to colonise isolated islands by rafting, direct development will have an advantage in maintaining a population within a restricted, isolated area.

Despite the enormous diversity of species in the Tropical Central Indo-Pacific, it is unlikely that this region is the fount from which clade B sprang, as the four species most basal in clade B occur on the Tropical East Pacific coastline, extending from Baja California to Peru. This aligns with the view that the richness of coral species in the Coral Triangle is due to 'range expansions into this region of species that evolved elsewhere' (Huang *et al.* 2017). This is in line with the view of Bowen *et al.* (2013, p. 235) that areas peripheral to biodiversity hotspots are "not evolutionary graveyards" but can export biodiversity and enrich other areas.

Southern Africa is a second area in which *Siphonaria* species have diversified, with 11 species in a relatively small area (see Chapter 2). This richness has a parallel in the patellid limpets, which have also radiated in the same region (Ridgway *et al.* 1998).

The unique oceanography of the region, with a juxtaposition of the warm southward-flowing Agulhas Current and the cold northward-moving Benguela Current, has created contrasting conditions and led to the recognition of multiple ecoregions around the coast (Emanuel *et al.* 1992, Sink *et al.* 2005) and an average of 34% endemism (Gibbons *et al.* 1999). Teske *et al.* (2011, 2019) has used a combination of these contrasting physical conditions, historical climate, thermal selection and larval dispersal rates to account for the high rates of speciation in limpets around the southern African coast. Australia has also been a focal point of radiation, supporting 10 species, split almost equally between clades A and B.

There are other obvious patterns in the biogeography of *Siphonaria* species. They are completely absent from high latitude Arctic and Antarctic realms (realms 1, 2 and 6 of Spalding *et al.* 2007, and realms 3, 4, 6 and 30 of Costello *et al.* 2017). They are also absent from the enclosed and freshwater-influenced Baltic and Black seas (Spalding's realms 2 and 7; Costello's realms 1 and 3). The Mediterranean, now also an enclosed sea, houses only a single native species, *S. pectinata*, despite the Tethys Sea having been suggested to be the point of origin of the family Siphonariidae (Hodgson 1999): a proposal that now seems unlikely in the light of this evidence. *Siphonaria pectinata* occurs on the west coast of Africa and the Canary Isles (but not Madeira or the Azores) and was originally confined to the western section of the Mediterranean, from where it has invaded eastwards (Gofas and Zenetos 2003, Delongueville and Scaillet 2010, Crocetta 2016, Slama *et al.* 2018). Additional invasions of *S. belcheri* and *S. crenata* have taken place in the eastern portions,

likely from the Arabian Sea and Red Sea (Albayrak and Çaglar 2006, Östürk *et al.* 2017). The first record of *S. crenata* arriving in the Great Bitter Lake connected to the Suez Canal was in 1906 (Tillier and Bavay, 1906). The near-absence of native species in the Mediterranean is a contrast with the fact that several fossil species are known from the Miocene Proto-Mediterranean (Harzhauser 2017), implying a loss of *Siphonaria* species since then, only to be boosted by alien arrivals and the spread of *S. pectinata*.

There are three cases of trans-hemispheric or trans-oceanic connections within clades. The existence of what was once thought to be a single species, *S. pectinata*, in both the eastern and western Atlantic is now known to be a case of genetically distinct sister species, *S. pectinata* and *S. naufragum*, in these respective regions (see above). Vermeij and Rosenberg (1993) have pointed out that these two regions have a long history of transoceanic exchanges, with the Western Atlantic probably the recipient of at least 17 Indo-West-Pacific species and 19 Eastern Atlantic species. They visualize that some species would have come from the Indian Ocean, first travelling westwards around southern Africa to the east coast of South America, and from there spreading eastwards to West Africa. This conforms with the maximally supported clade in my phylogeny that comprised these two species, with *S. capensis* as their sister taxon.

The division of clade A1 into a single species, *S. thersites*, in the cold far North Pacific, and three species from the Magellanic region of South America and the southern-ocean subantarctic islands is particularly intriguing, especially as the unnamed material from Kerguelen Island nests more closely with *S. thersites* than to the geographically closer *S. lateralis* and *S. fuegiensis*. The climatic barriers to dispersal between these northern and southern populations must be considerable, and there is no hint of human transfer between them. However, the group comprising *S. lateralis* and *S. fuegiensis* is relatively poorly supported (BP = 0.72) and if they were to form a grade rather than a clade, then one could argue that *S. thersites* may have originated by dispersal from the South.

Within what is considered one species, *S. zelandica*, there is a huge geographic hiatus between Western Australia and Japan, and the two sets sampled in these respective areas are genetically distinct – albeit sister taxa. This genetic differentiation was noted by Dayrat *et al.* (2014), but my addition of new material almost doubles the sample size and strengthens confidence that the difference is real, leading me to conclude that they may be a pair of cryptic species. Indeed, the very localised distributions of several units in the phylogeny of Dayrat *et al.* (2014), such as units 32 (Samoa) and 30 (Tonga), the unnamed species from Japan in my phylogeny, and species in the *S. nigerrima* complex described in Chapter 2, all point to the existence of many undescribed cryptic species within the genus.

Diversity relative to latitude

The genus *Siphonaria* is a useful one for exploring latitudinal trends because (a) there is a sufficiently large number of species (110 species including unnamed genetic units) to

validly seek trends; (b) they are a homogeneous group from morphological and evolutionary standpoints, so phylogenetic differences should not influence the outcome; (c) They are all ecologically equivalent, all being intertidal grazers, so that functional differences do not cloud patterns, and (d) they have an ancient history (Le Renard and Pacaud 1995, Pacaud and Le Renard 1995, Dayrat *et al.* 2011, Harzhauser *et al.* 2017) extending back to the Cretaceous with a major radiation in the Eocene, so their lineage is well established.

It has been a well-accepted principle that diversity (number of species) declines towards the poles, yielding a unimodal pattern with a peak at the tropics and a negative relationship between latitude and diversity towards the poles (Willig and Presley 2018). Willig *et al*. (2003) found this to be the case in ~70% of published analyses (with only ~10% each showing contrary positive, bimodal or non-existent relationships). In an analysis of nearly 200 marine studies, Hillebrand (2004) revealed significant average latitudinal declines in marine diversity, with the strength and direction of relationships being similar on land and in the sea, and stronger for regional (γ-diversity) than local (α-diversity) comparisons. This trend was general across hemispheres, ocean habitats and most organismal groups. Roy *et al.* (1994) showed that Eastern Pacific molluscs (2838 species, from Peru to Arctic) show a progressive decline in species richness with latitude, peaking at 5-10°N. The ranges of these species did not, however, show any correlation with latitude, contradictory to Rapoport's rule, and indicating that this cannot be the reason for the latitudinal trend.

There have, however, been challenges to the generality of this pattern of unimodal declines in diversity with latitude. Chaudhary *et al.* (2016) argue that a bimodal distribution of species richness vs. latitude is the norm in marine species, with the highest peak around 10-35°N (explained by greater availability of shallow-shelf habitat), a dip at the equator, and a second, smaller peak around 20°S.

Their conclusions have been challenged by Fernandez and Marques (2017) on the grounds that their composite analysis risks obscuring trends in different taxa and functional groups, does not consider the factors underlying latitudinal differences, and suffers from using a biased data set in which sampling is not uniform across latitudes. Countering this, Chaudhary *et al.* (2017) have conceded sampling bias, but showed that analyses that account for sampling intensity still reveal a dip in the tropics, although they even out the heights of the peaks to the north and south of the equator. They acknowledge that some taxa do indeed have a unimodal pattern, peaking in the tropics. For example, for predatory gastropods in the Eastern Atlantic, diversity is greatest in the tropics and there is a sudden increase in diversity at around 40°N where primary production changes from high-latitude seasonality to tropical continuous production (Taylor and Taylor 1977). Shumm *et al.* (2019) showed that for marine bivalves species richness and functional richness (diversity of functional types) both peak in the tropics and decline towards the poles. They also showed that bivalve species richness peaks longitudinally in the tropical

west Pacific (Coral Triangle), coinciding with the pattern that both Dayrat *et al.* (2014) and I have noted for *Siphonaria*.

On the other hand, in a study spanning 20-43°S in Chile, Rivadeneira *et al.* (2002) noted that diversity of herbivorous rocky-shore species peaked at mid-latitudes (ca 50 species at 30-32°S), and declined north and south of that area (ca 39 species at 18°S, 38 at 43°S). Similarly, Hernáez *et al.* (2020) detected a dip in the diversity of Western Atlantic burrowing shrimps (Axiidea and Gebiidea) at the equator, together with an asymmetry between the hemispheres with a higher peak in the north than the south.

My data for *Siphonaria* diversity relative to latitude accord with the view that diversity is bimodally distributed, declining towards the poles, but also exhibiting a dip at, and around, the equator and a higher peak in the northern than the southern hemisphere. There may be good ecological reasons why limpets in particular should decline in the tropics. The limpet shell shape is ill-adapted to counter desiccation and thermal stresses (Garrity 1984, Rivadeneira *et al.* 2002, Vermeij 2017), leading Branch (1985, p. 202) to comment "the limpet form is a disaster … in terms of heat gain and loss … This is perhaps one of the reasons why limpets decline in the tropics".

Geographic range relative to larval development

Thorson (1950, 1957) fathered the idea that at low latitudes benthic invertebrates primarily adopt a planktonic mode of larval development, with small eggs and larvae that have a relatively long life and a capacity for wide dispersion, whereas at high latitudes a progressively greater proportion embark on direct development, with large eggs and crawl-away larvae with more limited dispersal abilities. The most common explanation for this is that planktonic larvae will be at a disadvantage in cold waters because slow, prolonged development at low temperatures will expose them to greater risk of predation. Mileikovsky (1975) produced supporting evidence for this pattern and attached the name 'Thorson's law' to it. The concept has not, however, received universal support. Gallardo and Penchaszadeh (2001) found that the principle held among marine gastropods on the Pacific coast of South America, but not on the Atlantic coast, where benthic direct development predominates at all latitudes. Pearse (1994, p. 26) has demonstrated that cold-water echinoderms break the rule, and goes as far as suggesting that it should be "put to rest". Differences in the applicability of the rule exist among different groups of invertebrates, and between the hemispheres but, despite this, Marshall *et al.* (2012) concluded from a meta-analysis of over 1000 species that many aspects of Thorson's ideas are valid.

My data were limited in number because the larval development of the majority of *Siphonaria* species is unknown. But for those species for which the information does exist, there was strong support for the idea that low latitudes are typified by planktonic larval

development whereas high latitudes house a greater proportion of direct developers. The corollary is that domination of the tropics by planktonic dispersers, which have wide geographic ranges (Fig. 4.5) contradicts Rapaport's rule that latitudinal ranges of species are smaller at lower latitudes (Stevens 1989), joining a pool of other papers critical of the concept (Rohde *et al.* 1993, Roy *et al.* 1994, Rhode 1996, Gaston *et al.* 1998, Gaston and Chown, 1999), especially for marine systems (Tomasovych *et al.* 2016).

My analyses of both the phylogeny of *Siphonaria* and emergent patterns in their distribution has thus been a fertile field for exploring various ecological concepts.

Conclusions

My phylogeny reveals a deep division between clades A and B, which suggests that they could be classed as separate genera. However, as it is based on only one locus I would refrain from advocating a formal generic split. All the South African species, with the exception of *S. serrata*, are part of clade A. Therefore, the South African species do not form a monophyletic group, but the large majority constitute a local radiation.

A number of species, including all 11 southern African species, have been added to the current *Siphonaria* tree of Dayrat *et al.* (2014). Some of the unidentified units from the phylogeny of Dayrat *et al.* (2014) were identified here for the first time and assigned specific names.

I believe that there are about 110 described species or genetic units in the genus that can be considered as valid species (Appendix 4.2), although it is highly likely that many more new species will be recognized once a greater proportion of populations is analysed genetically. My analysis deals with 53 species or genetic units, so almost half the species remain to be analysed in this manner. Despite the deep phylogenetic division between the two major clades in the genus, I advocate retention of the name *Siphonaria* at the generic level to embrace the majority of species in the family Siphonariidae, rather than dividing it into genera that reflect the clades, at least until a sufficient number of species has been added to the phylogenetic tree to achieve taxonomic stability.

The genus *Siphonaria* is an ideal one to explore various ecological questions because it (a) comprises a phylogenetically, morphologically and ecologically uniform group, (b) contains a sufficiently large number of species to seek patterns, and (c) is widespread and occurs in most oceanic regions.

Drawing on information provided by my phylogeny and distributional records for the species, I was able to capitalize on the widespread occurrence of the genus to use it as a model to explore ecological correlates such as geographic range relative to mode of larval development, and variations in species diversity relative to latitude.

As I hypothesized, I was able to discern strong latitudinal trends in species richness within the genus, with high diversity present in the lower latitudes, in accord with data for many other marine taxa (Willig and Presley 2018), but with a marked dip at the equator –

which I attributed to the limpet shell form being poorly adapted for the stresses of tropical conditions (Vermeij 2017). I also found that species with direct larval development had significantly smaller latitudinal and geographic ranges than those with planktonic development, which is explicable in terms of their reduced dispersal ability (Chambers and McQuaid 1994).

I compared my phylogeny with the subgenera and 'sectia' proposed by Hubendick (1946) and conclude that although there is some correspondence between his divisions and the clades I detected, my phylogenetic analyses do not support retention of the majority of subgenera or sectia; many of these likely reflect convergence in shell morphology more than common ancestry. In short, like Dayrat *et al.* (2014) I showed that Hubendick's two subgenera are not natural monophyletic entities.

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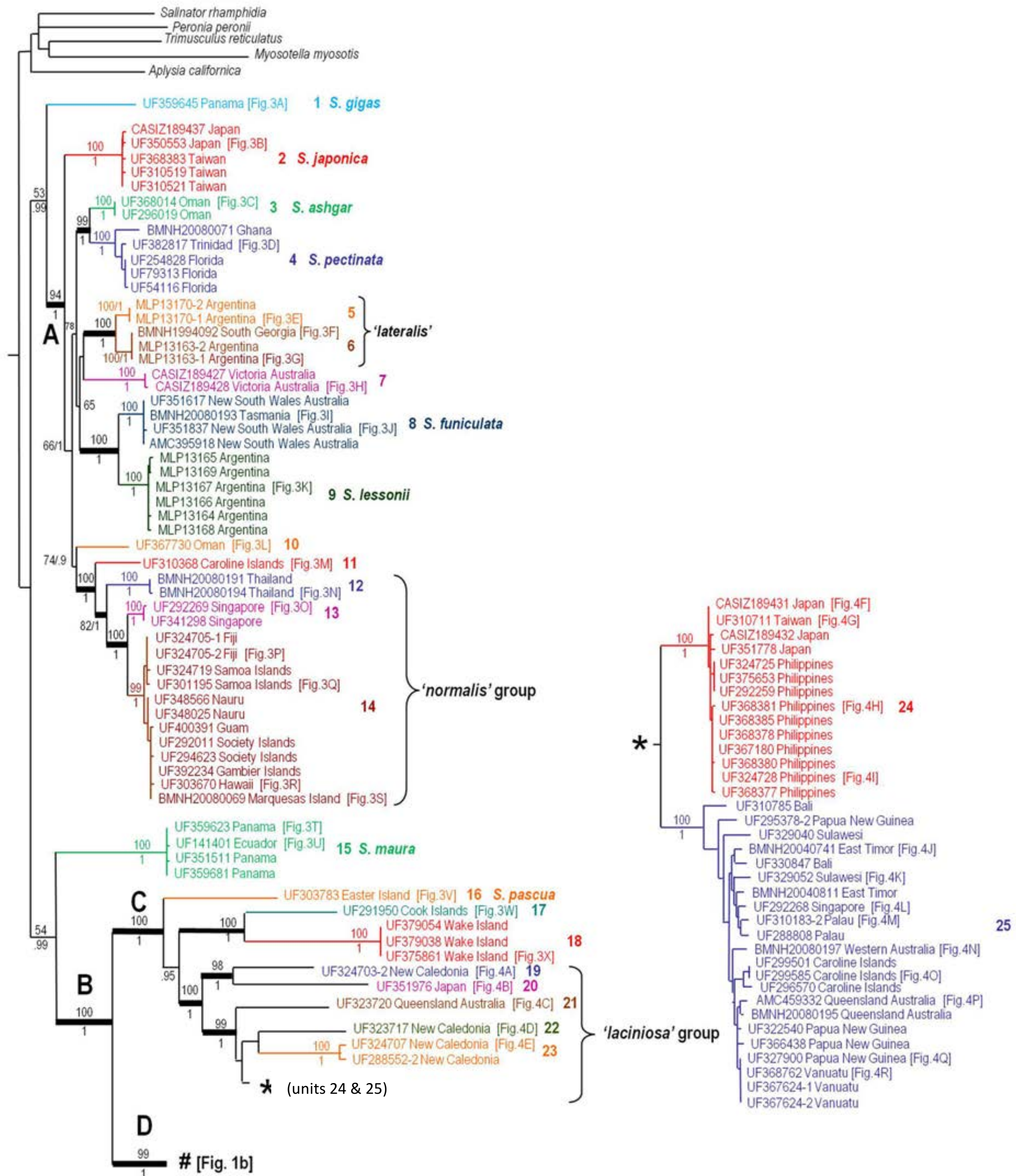
Appendix 4.1. Phylogeny of *Siphonaria* spp., reproduced from Dayrat *et al*. (2014, Figure 1, pp 249-250).



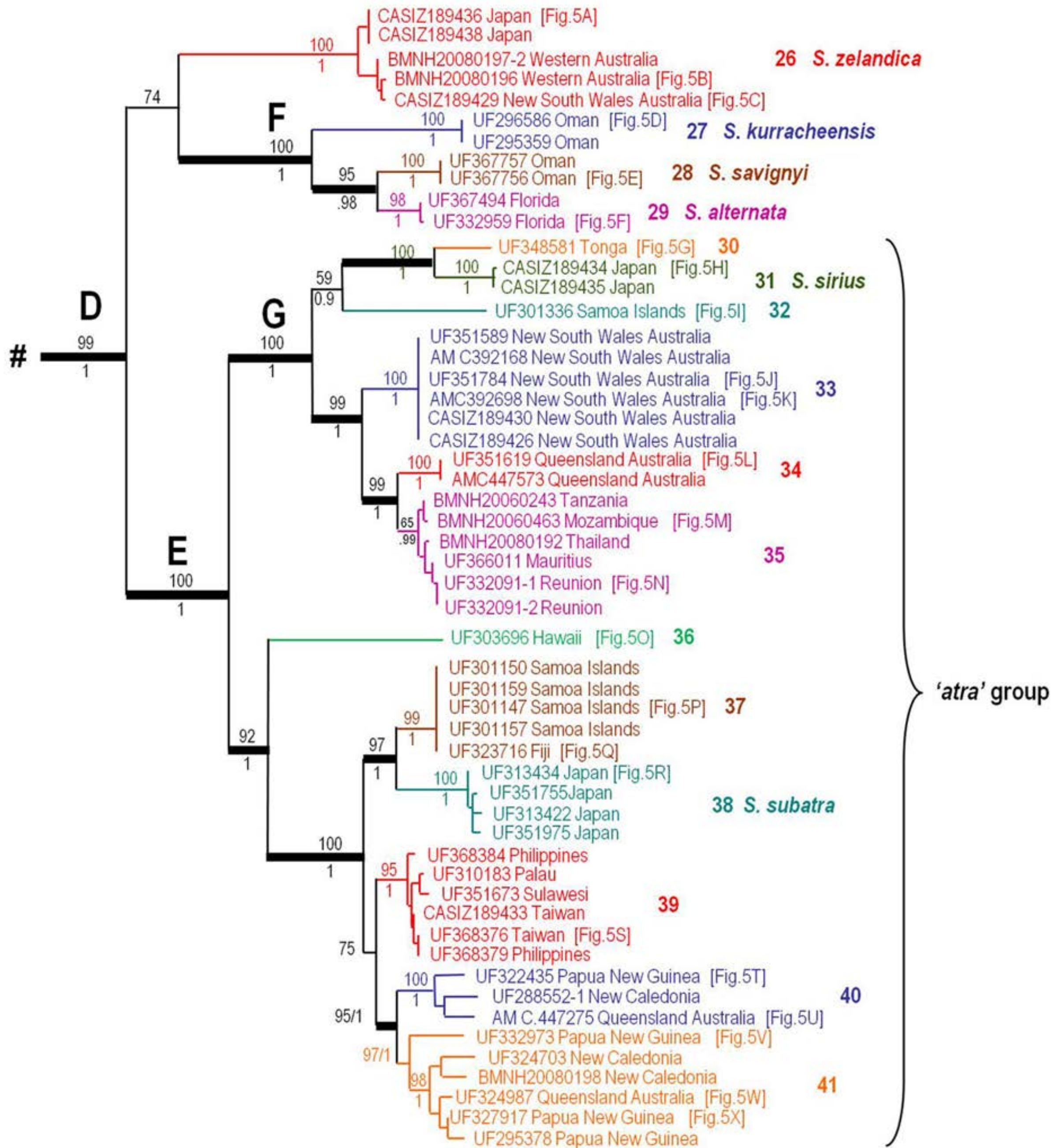

CASIZ189436 Japan [Fig.5A]
CASIZ189438 Japan
BMNH20080197-2 Western Australia
BMNH20080196 Western Australia [Fig.5B]
CASIZ189429 New South Wales Australia [Fig.5C]
26 S. zelandica
UF296586 Oman [Fig.5D]
UF295359 Oman
27 S. kurracheensis
UF367757 Oman
UF367756 Oman [Fig.5E]
28 S. savignyi
UF367494 Florida
UF332959 Florida [Fig.5F]
29 S. alternata
UF348581 Tonga [Fig.5G]
30
CASIZ189434 Japan [Fig.5H]
CASIZ189435 Japan
31 S. sirius
UF301336 Samoa Islands [Fig.5I]
32
UF351589 New South Wales Australia
AM C392168 New South Wales Australia
UF351784 New South Wales Australia [Fig.5J]
AMC392698 New South Wales Australia [Fig.5K]
CASIZ189430 New South Wales Australia
CASIZ189426 New South Wales Australia
33
UF351619 Queensland Australia [Fig.5L]
AMC447573 Queensland Australia
34
BMNH20060243 Tanzania
BMNH20060463 Mozambique [Fig.5M]
BMNH20080192 Thailand
UF366011 Mauritius
UF332091-1 Reunion [Fig.5N]
UF332091-2 Reunion
35
UF303696 Hawaii [Fig.5O]
36
UF301150 Samoa Islands
UF301159 Samoa Islands
UF301147 Samoa Islands [Fig.5P]
UF301157 Samoa Islands
UF323716 Fiji [Fig.5Q]
37
UF313434 Japan [Fig.5R]
UF351755Japan
UF313422 Japan
UF351975 Japan
38 S. subatra
UF368384 Philippines
UF310183 Palau
UF351673 Sulawesi
CASIZ189433 Taiwan
UF368376 Taiwan [Fig.5S]
UF368379 Philippines
39
UF322435 Papua New Guinea [Fig.5T]
UF288552-1 New Caledonia
AM C.447275 Queensland Australia [Fig.5U]
40
UF332973 Papua New Guinea [Fig.5V]
UF324703 New Caledonia
BMNH20080198 New Caledonia
UF324987 Queensland Australia [Fig.5W]
UF327917 Papua New Guinea [Fig.5X]
UF295378 Papua New Guinea
41
'atra' group
#
D
E
F
G
99
1
74
100
1
100
1
95
.98
98
59
0.9
99
65
.99
92
97
75
95/1
97/1

FIGURE 1. Phylogenetic tree obtained based on concatenated CO1, 12S, and 16S sequences of 154 individuals of Siphonaria. Maximum likelihood and Bayesian analyses yielded identical topologies. MCMC posterior probabilities (superior to 0.9) and bootstrap values (superior to 50%) are indicated for each node. Each individual is referred to by its catalogue number in its corresponding museum collection. The shells of some individuals are illustrated on plates (Figs 3–5) and illustrations are indicated here between brackets. Each illustration corresponds to the exact individual from which DNA was actually extracted. The 41 molecular units recognized here are indicated in different colors. Some units can be named unambiguously, such as *Siphonaria gigas* (unit 1), but some other units can only be placed within a species group where units are hardly distinguishable conchologically, such as the '*laciniosa*' group (units 19 to 25). See the text for more details. Bold, capital letters A to G indicate some supraspecific nodes that are discussed in the text.

Appendix 4.2A

List of species currently recognized by MolluscaBase in the genus *Siphonaria*, or which I consider should be placed in the genus: names (bold if included in my phylogeny), geographic distribution, notes on synonymy, unit numbers allocated in Dayrat *et al.* (2014), and references for distribution (see Appendix 4.2B). The lower portions of the table list unnamed genetic units and new species; synonyms of accepted species; and accepted fossil (extinct) species. 'Accepted as' indicates acceptance by MolluscaBase unless otherwise stated.

Living species accepted as valid in this thesis (bold if included in phylogeny)	Type locality or geographic distribution	Synonyms & notes	Dayrat et al. (2014) Unit No	References for distribution
Siphonaria acervus (Iredale, 1940)	Anala, New Caledonia	Accepted as *Triellsiphon acervus* Iredale, 1940		47
Siphonaria acmaeoides Pilsbry, 1895	Japan; S Korea	*S. a. paulae* Christiaens, 1980 is recognised as a subsp.		22, 77
Siphonaria acuta Quoy & Gaimard, 1833	Indonesia & Solomon Islands, Papua New Guinea			80, 82
Siphonaria aequilirata Carpenter, 1857	Mazatlán, Gulf of Mexico			–
Siphonaria aequilorata Reeve, 1856	Mazatlán, Gulf of Mexico; Margarita Bay & Is.; Tres Marías Is			67, 83
Siphonaria alba Hubendick, 1943	Near Singapore, Java Sea			43
Siphonaria albicante Quoy & Gaimard, 1833	Soloman Islands & Papua New Guinea.	Possibly synonym of *S. laciniosa* (Linnaeus, 1758) (WMSDB)		80
***Siphonaria alternata* (Say, 1826)**	East Florida; also E&W coasts of Panama; Bahamas; Bermuda		*29*	64, 67, 71, 83
Siphonaria alternicosta Potiez & Michaud, 1838	Unknown type locality			101
***Siphonaria anneae* Tomlin, 1944**	KwaZulu-Natal, South Africa	Incorrectly accepted as *S. carbo*. *S. anneae* considered a valid species: Chapter 2		96, 102
***Siphonaria asghar* Biggs, 1958**	Hormuz Island, Persian Gulf, Emirates.	Resembles *S. tenuicostulata*	*3*	8, 10, 12, 26
***Siphonaria atra* Quoy & Gaimard, 1833**	Solomon Is, Philippines; Japan, Vanikoro Island, N Australia		*32-41*	1,22, 47, 59, 80
***Siphonaria australis* Quoy & Gaimard, 1833**	New Zealand			6, 50, 78, 80
Siphonaria basseinensis Melvill, 1893	Bombay, India			62, 99
***Siphonaria belcheri* Hanley, 1858**	Levantine Sea, E Mediterranean; alien			2, 7, 8, 12, 30, 40, 76
Siphonaria bifurcata Reeve, 1856	Philippine Islands			50, 99
***Siphonaria brannani* Stearns, 1873**	Santa Barbara Is, California; Baja California, Mexico			54, 67
Siphonaria brasiliana Reeve, 1856	Rio Janeiro, Brazil			99
Siphonaria brunnea Hanley, 1858	Bermuda	Regarded by gastropods.com as synonym of *S. alternata* (Say, 1826)		40
***Siphonaria capensis* Quoy & Gaimard, 1833**	Cape of Good Hope, South Africa			3, 15, 56, 80, 84, 102
Siphonaria carbo Hanley, 1858	?Caribbean ?Oman	Not applicable to South African *S. nigerrima* material (this thesis, Chapter 2)		12, 17
Siphonaria characteristica Reeve, 1842	No locality for type; possibly Panama	Possible synonym of *S. gigas* Sowerby, 1824		–
Siphonaria chirura Pilsbry, 1920	Hawaii	Treated as a subsp of *S. normalis* by WMSDB		99
***Siphonaria compressa* Allanson, 1958**	Langebaan, South Africa	Regarded as distinct from New species 3 (this thesis, Chapter 3)		3, 5
***Siphonaria concinna* G. B. Sowerby I, 1823**	South-east South Africa	Accepted as *S. concinna*, distinct from *S. oculus* (this thesis, Chapter 2		3, 15, 41, 56, 102
Siphonaria corallina Christaens, 1980	Hong Kong.	*Kerguelenella corallina* (Christaens, 1980)		22
Siphonaria coreensis A. Adams & Reeve, 1848	Corean Peninsula, Korea	Listed by gastropods.com as synonym of *S. laciniosa* (Linnaeus, 1758)		99
Siphonaria corrugata Reeve, 1856	Island of Luzon, Philippines			82
Siphonaria corrumbinensis Hubendick, 1955	Queensland			44
Siphonaria costata G. B. Sowerby I, 1835	Central America; Baja California, Mexico			88
***Siphonaria crenata* de Blainville, 1827**	Locality unspecified; likely Gulf of Aqaba, Red Sea			7, 27, 30, 35, 76, 95

***Siphonaria denticulata* Quoy & Gaimard, 1833**	Port Western; Victoria, W Australia, Queensland			24, 80
***Siphonaria diemenensis* Quoy & Gaimard, 1833**	Tasmania, S Australia, NSW			6, 60, 80
Siphonaria exulum Hanley, 1858	Norfolk Island Australia	Synonym of *S. diemenensis* Quoy & Gaimard, 1833 (a/t Sealifebase.ca)		40
Siphonaria ferruginea Reeve, 1856	Type locality not specified; Mauritius, Reunion			31
***Siphonaria fuegiensis* Güller, Zelaya & Ituarte, 2016**	Tierra de Fuego, Malvinas (Falklands)		*5*	36, 39
***Siphonaria funiculata* Reeve, 1856**	Tasmania, NSW, Queensland, W Victoria		*8*	6, 24
***Siphonaria gigas* G. B. Sowerby I, 1825**	Panama Pacific Coast		*1*	57, 67
Siphonaria guamensis Quoy & Gaimard, 1833	Port d'Humata, Guam, Singapore, Marianas, West Pacific; Fiji			21, 43, 80
Siphonaria henica Verrill & Bush, 1900	Bailey Bay, Bermuda Islands			99
Siphonaria hispida Hubendick, 1946	Rio de Janeiro Brazil, S Brazil; Isle Fernando			29, 61
Siphonaria incerta Deshayes, 1863	Probably Reunion Islands			99
Siphonaria innocuus (Iredale, 1940)	Norfolk Island, Australia	Probable synonym of *S. zelandica* Quoy & Gaimard, 1833		47
Siphonaria innominata (Iredale, 1915)	Antipodes, Auckland, Macquarie, Campbell sub-Antartic Islands	Possibly a synonym of *S. lateralis*		46, 99
***Siphonaria japonica* (Donovan, 1824)**	Japan; Hong Kong		*2*	1, 22, 42, 66, 69, 73
Siphonaria javanica (Lamarck, 1819)	Indonesia, Thailand, Philippines			16, 99
***Siphonaria jeanae* Jenkins, 1984**	Ceduna, SW Australia; south-east Australia			6, 51
***Siphonaria kurracheensis* Reeve, 1856**	Karachi Pakistan, also Red Sea, N Mozambique	Australian specimens incorrectly assigned this name		8, 12, 69, 94
***Siphonaria laciniosa* (Linnaeus, 1758)**	India, Indonesia, Philippines		*19-25*	16, 22, 45, 47, 58, 59
Siphonaria laeviuscula G. B. Sowerby I, 1835	Valparaiso, Chile	May be the equivalent of N Chile/Peru '*S. lessonii*'		88
***Siphonaria lateralis* Gould, 1846**	Tierra del Fuego; Falklands, sub-Antarctic islands	Placed *Kerguelenella* by some authors but accepted as *S. lateralis*	*6*	14, 28, 38, 39, 67, 85, 87
Siphonaria lecanium Philippi, 1846	Acapulco, Mazatlán, Manzanillo, Mexico; Baja California			67, 99
Siphonaria lentulus (Iredale, 1940*)*	Lord Howe Island	Accepted as *Mestosiphon lentulus* Iredale, 1940 by MolluscaBase		47
Siphonaria lepida Gould, 1848	Rio de Janiera, Brazil			99
***Siphonaria lessonii* Blainville, 1827**	Falkland Islands, Argentina, Chile, Punta del Esta Uruguay		*9*	36, 39, 67, 68, 75
Siphonaria lineolata G. B. Sowerby I, 1835	Chiloe Island, Chile; Peru			88
Siphonaria macgillivrayi Reeve, 1856	Island of St Paul's, Indian Ocean; Magellanic Region(?)	Considered to be a synonym of *S. fuegiensis* by Guller *et al.* (2016)		82
Siphonaria macquariensis (Powell, 1939)	Macquarie Island, Stewart Island Australia	Accepted as *Kerguelenella macquariensis* (Powell, 1939) by MolluscaBase		78
Siphonaria madagascarensis Odhner, 1919	Majunga, Madagascar, Grande Comore			72
Siphonaria marza (Iredale, 1940)	NSW to Port Douglas, Queensland	Ellsiphon marza Iredale 1940; Provisionally placed in *Siphonaria*.		47
***Siphonaria maura* G. B. Sowerby I, 1835**	Nicaragua, Panama , Ecuador, Central Pacific Coasts		*15*	67, 88
Siphonaria monticulus (Iredale, 1940)	Loyalty Islands, New Caledonia	Accepted in genus *Hebesiphon* by MolluscaBase, but not by Hubendick 1946		47
***Siphonaria naufragum* Stearns, 1872**	Amelia Is, Florida	Valid, but previously synonymised with *S. pectinata* (Linnaeus, 1758)		34, 53, 67, 71, 83, 89,98
***Siphonaria nigerrima* Smith, 1903**	Umhlali, KwaZulu-Natal, South AfricaNatal	To replace the name S. carbo as applied to SA material (this thesis, Chapter 2)		19, 20, 92, 102
***Siphonaria normalis* A. A. Gould, 1846**	Sandwich Is Hawaii; Indo-Pacific, Réunion, Thailand, Hawaii	Hawaiian (type locality) material looks different	*12, 13, 14*	37, 40, 54, 80
***Siphonaria oculus* F. Krauss, 1848**	Table Bay, South Africa			15, 41, 56, 102
Siphonaria oppositus (Iredale, 1940*)*	Queensland, Port Curtis, Fiji	Accepted as *Malorisiphon oppositus* Iredale, 1940. Possibly = *S. laciniosa*		47
Siphonaria parcicostata Deshayes, 1863	No type locality specified; probably Reunion Is			99
Siphonaria parma Hanley, 1858	West Africa	Accepted species, but distribution suggests = *S. pectinata*		99
***Siphonaria pascua* Rehder, 1980**	Easter Island		*16*	23, 81
***Siphonaria pectinata* (Linnaeus, 1758)**	Mediterranean, Canary Islands, West Africa		*4*	25, 32, 33, 34, 35, 70, 86, 100
Siphonaria percea (Iredale, 1940)	Cook Islands			99
Siphonaria pica G. B. Sowerby I, 1835	Acapulco, Mexico; Sea of Cortez, W Mexico- Ecuador	Synonymised as *S. maura* by Hubendick (1946)		99
Siphonaria pisangensis Hubendick, 1947	Isle of Pisang, West Papua, Indonesia			99
Siphonaria placentula Menke, 1853	No type locality specified; likely Cape Verde	Name applied to Cape Verde Islands members of '*S. pectinata*'		34, 53, 63
Siphonaria plicata Quoy & Gaimard, 1833	Hihifo, Tonga	Listed by gastropods.com as synonym of *S. laciniosa* (Linnaeus, 1758)		80, 99

Siphonaria propria Jenkins, 1983	Kaikoura Peninsula, New Zealand			50
Siphonaria punctata Quoy & Gaimard, 1833	Port Louis, Mauritius			80, 99
Siphonaria raoulensis W. R. B. Oliver, 1915	Kermadec Islands and N Islands, New Zealand			74
Siphonaria redimiculum Reeve, 1856	No type locality	Probably = *Siphonaria lateralis* Gould, 1846		82
Siphonaria rosea Hubendick, 1943	Kharg Is, Iran, Persian Gulf			99
Siphonaria rucuana Pilsbry, 1904	Ryukyu Islands, Japan			99
***Siphonaria savignyi* Krauss, 1848**	Type locality not specified, probably Red Sea; Oman		*28*	12, 56, 99
***Siphonaria serrata* (Fischer von Waldheim, 1807)**	Type locality not specified; probably Cape of Good Hope	Previously named *S. aspera* Krauss, 1848		4, 15, 41, 56, 84, 102
Siphonaria siquijorensis Reeve, 1856	Island of Siquijor, Philippines			99
***Siphonaria sirius* Pilsbry, 1895**	Thailand to Philippines, S Japan; Indonesia, Korea		31	16, 22, 48, 49, 59, 77
***Siphonaria soranus* (Iredale, 1940)**	Port Townsville, Queensland	Accepted as *Planesiphon soranus* Iredale, 1940 in MolluscaBase		47
Siphonaria stellata (Helbling, 1779)	no type locality			–
Siphonaria stewartiana (Powell, 1939)	Stewart Island, South Island, New Zealand.	Originally described as *Kerguelenia stewartiana* Powell, 1939		55, 78
***Siphonaria subatra* Pilsbry, 1904**	Bonin Islands, Japan; Taiwan, Philippines			22, 99
Siphonaria subrugosa G. B. Sowerby I, 1835	Coast of Brazil	Regarded by gastropods.com as synonym of *S. hispida*		88
***Siphonaria tasmanica* Tenison Woods, 1876**	Tasmania, Victoria, New South Wales, Queensland W Australia	Described as *Talisiphon tasmanicus* Tenison Woods, 1876		6, 79, 91
***Siphonaria tenuicostulata* Smith, 1903**	Umhlali, KZN, South Africa	Currently synonymised as *S. carbo*, but resurrected in this thesis, Chapter 2		17, 18, 19, 20, 102
Siphonaria tenuis Philippi, 1860	Central Chilean coast, 30-40°S			99
***Siphonaria thersites* Carpenter, 1864**	Neeah Bay; Washington State to Aleutian Islands			13, 67, 90, 97
Siphonaria tongensis Hubendick, 1943	Tonga			99
Siphonaria tristensis G. B. Sowerby I, 1823	Type locality not specified, but occurs at Tristan Islands			39, 99
Siphonaria venosa Reeve, 1856	Cape Coast, Ghana	Almost certainly = *S. pectinata* (WMSDB & personal view)		82
Siphonaria viridis Quoy & Gaimard, 1833	Ambon, Moluccas Islands, Indonesia, New Guinea			80
Siphonaria williamsi Berry, 1969	Baja California, Mexico		*26*	9
***Siphonaria zelandica* Quoy & Gaimard, 1833**	New Zealand & Keppel Bay to Broome, E Australia			6, 47, 50, 60, 66,80
Unnamed or new species/units				
moz115Tofo, Moz117Tofo, Q112Tofo	New sp 1; Tofo, Mozambique			102
QMoz429Mkb, Q181Mkb	New sp 2; Mkambati, E Coast of South Africa			102
Knysna new sp 3	New sp 3: Knysna Lagoon, South Africa			4, 102
Taiwan372PngHu, Japan403Ogswra, 404Ogswra, E1403077	Unnamed sp a, Japan			103
kur145Rtn, kur146Rtn, kur147Rtn	Unnamed sp b Rottnest Is; probably widespread in Australia			6
Unit 7	Unnamed sp; Victoria, Australia		*7*	26
Unit 10	Unnamed sp. d; Oman		*10*	26
Unit 11	Unnamed sp; Caroline Is		*11*	26
Unit 17	Unnamed sp; Cook Islands		*17*	26
Unit 18	Unnamed sp; Wake Island		*18*	26
Unit 24	Unnamed sp; Japan, Taiwan		*24*	26
Unit 25	Unnamed sp; Borneo, Indonesia, W Australia		*25*	6, 11, 52
Unit 27	Oman	Probably incorrectly named *S. kurracheensis*	*27*	26
Unit 30	Tonga	'atra' group	*30*	26

Synonymised or questionable *Siphonaria* species	Distribution	Current status (MolluscaBase)
Siphonaria adansonii (Blainville, 1824)	Senegal	Accepted as *S. pectinata* (Linnaeus, 1758)
Siphonaria adjacens Turton, 1932	Pt Alfred, South Africa	Taxon inquirendum; = S. concinna or S. oculus, this thesis Chapter2
Siphonaria albofasciata Krauss, 1848	Cape' South Africa	Accepted as *S. concinna* G. B. Sowerby, 1823.
Siphonaria algesirae Quoy & Gaimard, 1833	Strait of Gibraltar)	Accepted as *S. pectinata* (Linnaeus, 1758
Siphonaria alterniplicata Grabau & King, 1928	Possibly Beidaihe, China	Accepted as *S. japonica* (Donovan, 1824)
Siphonaria amara Reeve, 1856	California but more likely Hawaii	Accepted as *S. normalis* Gould, 1856
Siphonaria amphibia Oliver, 1915	Kermadec Islands	Accepted as *S. raoulensis* Oliver, 1915
Siphonaria angulata Gray, 1927	Unknown locality	Taxon inquirendum
Siphonaria anneae Tomlin, 1944.	Umpangazi, KZN, South Africa	Accepted as S. carbo; resurrected as S. anneae in this thesis, Chapter 2
Siphonaria antarctica Gould 1852	Southern S America	Accepted as *S. lessonii* de Blainville, 1827
Siphonaria ashgar	Persian Gulf, Emirates	Unknown author, nomen lapsus; accepted as *S. asghar* Biggs, 1958
Siphonaria aspera Krauss, 1848.	Cape to Natal	Accepted as *S. serrata* (Fischer von Waldheim, 1807)
Siphonaria atrata Quoy & Gaimard, 1933	Philippines	Not listed by MolluscaBase. Probably *S. laciniosa* (Linnaeus, 1759)
Siphonaria baconi Reeve, 1856	Swan River, Western Australia	Accepted as *S. zelandica* Quoy & Gaimard, 1833
Siphonaria becki Turton, 1932	Port Alfred, South Africa	Taxon inquirendum, accepted as S. oculus in this thesis, Chapter 2
Siphonaria blainvillei Hanley, 1858	No type locality	Accepted as *S. funiculata* Reeve, 1856
Siphonaria bifurcata Reeve 1856	Philippine Islands	Listed by some as *S. zelandica* Quoy & Gaimard, 1833
Siphonaria cancer Reeve, 1856	No type locality; New Zealand	Accepted as *S. australis* Quoy & Gaimard, 1833
Siphonaria cheesemani Oliver, 1915	Kermadec Islands	Accepted as *S. raoulensis* Oliver, 1915
Siphonaria chirura Pilsbry, 1920	Hawaii	Accepted as *S. chirura* or as a subspecies of *S. normalis*
Siphonaria cochleariformis Reeve, 1856	Isle of Hainan, Coast of China	Nomen dubium; Accepted as *S. japonica* Reeve, 1856
Siphonaria comita Iredale, 1924	New South Wales	Accepted as variety of *Pugilaria stowae* (Verco, 1906)
Siphonaria commixtus (Iredale, 1940)	New Caledonia	Taxon inquirendum described as *Parellsiphon commixtus* Iredale, 1940
Siphonaria conica Blainville,1827	No type locality	Taxon inquirendum; probably S. pectinata (Linnaeus, 1758)
Siphonaria cookiana Suter, 1909	Cook Strait, New Zealand	Accepted as *S. australis* Quoy & Gaimard, 1833
Siphonaria cornuta Gould, 1848	Mangsee Islands, Philippines, = *S. javanica*	Accepted as *S. javanica* (Lamarck, 1819)
Siphonaria corrumbinensis Hubendick, 1955	E Australia	Accepted as *S. laciniosa* (Linnaeus, 1758)
Siphonaria cyaneomaculata Sowerby GB, 1906	'Cape' South Africa	Accepted as *S. concinna* Sowerby, 1823
Siphonaria dayi Allanson, 1958	Inhaca Island	Synonymised as S. nigerrima Smith, 1903; this thesis, Chapter 2
Siphonaria densata (Iredale, 1940)	N Queensland	*= Legosiphon densatus*; Nomen dubium; accepted as *S laciniosa*
Siphonaria deflexa Helbling, 1779	Cape, South Africa	Nomen dubium, accepted as *S. concinna* Sowerby, 1823
Siphonaria depressa Pease, 1862	Type locality not specified: Central Pacific Islands	Taxon inquirendum; likely synonym of *S. pectinata*
Siphonaria depressior Schrenck, 1867	Hokkaido Japan	Accepted as subsp. of *S. sipho*; now = *S. javanica*
Siphonaria elatior Schrenck, 1940	Philippines	Subsp. of *S. sipho*, now accepted as *S. javanica*
Siphonaria elegans (Iredale, 1940)	Keppel Bay Queensland	*= Planesiphon elegans*, accepted as *S. zelandica*
Siphonaria eumelas (Iredale, 1940)	Snapper Is. Queensland	Accepted as *S. laciniosa* (Linnaeus, 1758).
Siphonaria exigua Sowerby I, 1823	No type locality	Taxon inquirendem; var of *S. laciniosa* a/t Hubendick 1946
Siphonaria exulorum	Norfolk Island	Incorrect spelling of *S. exulum* Hanley, 1858
Siphonaria flemingi (Powell, 1955)	Auckland Islands, New Zealand	Accepted as *S. innominata* (Iredale 1915)
Siphonaria fuliginata Reeve,1856	No type locality	Nomen dubium
Siphonaria grisea (Gmelin, 1791)	West Africa	Accepted as *S. pectinata* (Linnaeus, 1758)

Siphonaria inculta Gould, 1848	New Zealand	Accepted as *S. australis* Quoy & Gaimard, 1833.
Siphonaria intermedia CA Davis 1904	Bermuda; not in MolluscaBase,	Regarded as synonym of *S. alternata* (Say, 1827)
Siphonaria intermedia Schrenck, 1967	Philippines	Accepted as *S. javanica* Lamarck, 1819)
Siphonaria jonasi Dunker, 1853	Unknown locality	Accepted as *S. pectinata* (Linnaeus, 1758)
Siphonaria kowiensis Turton, 1932	Kowie, South Africa	Worn specimen. Considered nomen dubium in this thesis, Chapter 2
Siphonaria laevis Philippi, 1846	Chile	Nomen dubium; probably synonym of *S. lessonii*
Siphonaria lentulus (Iredale, 1940)	Lord Howe Island	Accepted as synonym of *S. zelandica* Quoy & Gaimard, 1833
Siphonaria leucopleura (Gmelin, 1791)	No type locality specified	Described as a *Patella*, but now assigned to *Lottia*
Siphonaria lineata Lamarck, 1891	No type locality specified; Ecuador, Chile	Incorrect spelling of *S. lineolata*
Siphonaria lineolata d'Aubigny, 1843	Florida	Accepted as *S. naufragum* Stearns, 1872
Siphonaria lirata Reeve, 1856	No type locality specified	Accepted as *S. normalis* A. A. Gould, 1846.
Siphonaria luzonica Reeve, 1846	Island of Luzon, Philippines	Taxon inquirendem
Siphonaria macauleyensis Oliver, 1915	Kermadec Islands, New Zealand	Accepted as *S. raoulensis* Oliver, 1915
Siphonaria macquariensis (Powell, 1939)	Macquarie Island, Australia	Accepted as *Kerguelenella macquariensis* (Powell, 1939)
Siphonaria magellanica Odhner, 1919	No type locality, possible Straits of Magellan	Accepted as *S. lessonii* de Blainville, 1827
Siphonaria marza Sowerby I, 1835	NSW to Port Douglas; Keppel Bay, Queensland	Accepted as *Ellsiphon marza* Iredale, 1940
Siphonaria melanoleuca (Gmelin, 1791)	No type locality. Possibly Senegal	Probably *S. pectinata* (Linnaeus, 1758)
Siphonaria melanozonias (Gmelin, 1791)	No Type locality	Taxon inquirendum
Siphonaria milneedwardsi Locard, 1898	Praia, Cape Verde	Accepted as *S. placentula* Menke, 1853
Siphonaria minor Pallary, 1900	Oran, Algeria	Subsp of *S. mouret*, accepted as *S. pectinata* (Linnaeus, 1758)
Siphonaria mirificus (Iredale, 1940)	Magnetic Is, Townsville, Queensland	Originally *Legosiphon mirificus*. Accepted as *S. laciniosa*
Siphonaria mouret Sowerby I, 1825	W Africa	Accepted as *S. pectinata* (Linnaeus, 1758)
Siphonaria mouretus Blainville, 1824)	No type locality, probably Dakar, Senegal	Accepted as *S. pectinata* (Linnaeus, 1758)
Siphonaria mulinus (Iredale, 1940)	Noumea, New Caledonia	Taxon inquirendum. Described as *Legosiphon mulinus* Iredale, 1940.
Siphonaria natalensis Krauss, 1848	SE Coast, South Africa	Regarded as S. serrata (Fischer von Waldheim, 1807); this thesis, Chapter 2
Siphonaria nereis (Iredale, 1940)	Victoria Australia; subsp of *S. tasmanicus*	Accepted as *Siphonaria tasmanica* Tenison Woods, 1876
Siphonaria nigra Pallary, 1900	Oran, Algeria	Subsp of *S. mouret*, accepted as *S. pectinata* Linnaeus (1758)
Siphonaria nuttallii Hanley, 1858	Hawaiian Islands	Accepted as *S. normalis* AA Gould, 1846
Siphonaria oblivirgulata Hubendick, 1943	Unknown locality	Accepted as *Siphonaria funiculata* Reeve, 1856
Siphonaria opalescens Davis, 1904	Bermuda	Taxon inquirendum, but regarded as *S. alternata* (gastropods.com)
Siphonaria oppositus (Iredale, 1940)	Queensland, Port Curtis, Fiji	'atra' group; suggested = *S. laciniosa* (WMSBD) or *S atra* (Sealifebase)
Siphonaria optiva (Iredale, 1940)	Magnetic Is, Queensland	= *Legosiphon optivus* Iredale, 1940; Accepted as *S. laciniosa*
Siphonaria pallida Allanson, 1958	Langebaan Lagoon, South Africa	Taxon inquirendum; variety of S. serrata in this thesis, Chapter 2
Siphonaria palmata Carpenter 1857	Mazatlán, Sea of Cortez, W Mexico	Taxon inquirendum; treated as form of *S. maura* by gastropods.com
Siphonaria palpebrum Reeve, 1856	Lisbon, Portugal	Probably synonymous with *S. pectinata* (Linnaeus, 1758)
Siphonaria pampa Ihering, 1914	Argentina	Accepted as *S. lessonii* Blaineville, 1827 by WMSDB gastropods.com
Siphonaria parmelas (Iredale, 1940)	New Caledonia	Taxon inquirendum; possibly = S. laciniosa
Siphonaria picta d'Orbigny, 1839	No type locality specified; Fernando Noronha Is Brazil; Cuba	Taxon inquirendum; possibly = S. naufragum (WMSDB) or S. hispida
Siphonaria plana Quoy & Gamard, 1833	Port Louis, Mauritius; nomen dubium	Nomen dubium
Siphonaria promptus (Iredale, 1940)	Capricorn Islands, Queensland	= *Parellsiphon promptu*s Iredale, 1940; Accepted as *S. laciniosa*
Siphonaria radians Adams & Adams, 1855	Type locality not specified	Accepted as *S. japonica* Donovan, 1824)
Siphonaria radiata Blainville, 1827	Gambia	Nomen dubium; probably synonymous with *S. pectinata* (Linnaeus, 1758
Siphonaria radiata Gray, 1824	Type locality not specified	Taxon inquirendum; junior subjective synonym for *S. concinna/exigua/sipho*
Siphonaria scabra Reeve, 1856	Sydney, NSW, Australia	Taxon inquirendum; probably = *S. diemenensis* Quoy & Gaimard, 1833

Siphonaria scutellum Deshayes, 1841	Chatham Islands, New Zealand	Accepted as *Benhamina obliquata* (G. B. Sowerby I, 1825)
Siphonaria sipho Sowerby, 1823	Type locality not specified	Accepted as *S. javanica* (Lamarck, 1819). Type species of *Siphonaria*.
Siphonaria sowerbyi Michelin, 1832	Type locality unknown	Taxon inquirendum; possibly *S. pectinata* (Linnaeus, 1758)(WMSDB)
Siphonaria spinosa Reeve, 1856	Putatively New Zealand, but very unlikely	Accepted as *S. serrata* (Fischer von Waldheim, 1807)
Siphonaria stellata Blainville, 1827	no type locality	Accepted as *S. laciniosa* (Linnaeus, 1758)
Siphonaria stowae Verco, 1906	SE Australia	Synonym of Pugillaria stowae (Verco, 1906)
Siphonaria striatocostata Dunker, 1846	West Africa	Accepted as *S. pectinata* (Linnaeus, 1758)
Siphonaria tenuis Philippi, 1860	Chile	Synonym of *S. lessonii* de Blainville, 1827 a/t gastropods.com
Siphonaria umbonata Menke, 1853	Cape Verde?	Accepted as *S. placentula* Menke 1853
Siphonaria variabilis F. Krauss, 1848	SE coast of South Africa	Accepted as *S. concinna* Sowerby, 1823
Siphonaria virgulata Hedley, 1915	Sydney, NSW, Australia	Accepted as *S. funiculata* Reeve, 1856
Siphonaria zanda (Iredale, 1940)	Low Isles, N Queensland	= *Parellsiphon zanda* Iredale, 1940; Accepted as *S. laciniosa*
Siphonaria zebra Reeve, 1856	Chatham Islands, New Zealand	Accepted as *S. zelandica* Quoy & Gaimard, 1833
Siphonaria zonata Tenison-Woods, 1878	S Tasmania & Victoria	Objective synonym of *S. tasmanica* Tennison Woods, 1978
Fossil species		
Anisomyon borealis Meek & Hayden, 1860	Alabama, USA	Not listed by Molluscabase
Anisomyon centrale FB Meek, 1871	Alberta, Canada	Not listed by Molluscabase
Anisomyon patelliformis Meek & Hayden, 1860		
Anisomyon shumardi Meek & Hayden, 1860		
Anisomyon vectis Gardner, 1877	Isle of Wight	
Siphonaria bisphites Michelin, 1831	France	= *S. subcostaria* (A. d'Orbigny, 1852) and *S. variecostata* Sacco, 1897
Siphonaria costaria (Dehayes, 1824)	France	
Siphonaria crassicostata Deshayes, 1861	France	
Siphonaria glabrata de Raincourt, 1876	France	
Siphonaria granicosta (Cossman, 1895)	Brittany, France	
Siphonaria irregularis Sacco, 1897		
Siphonaria laubrierei Cossman, 1889	France	
Siphonaria liancurtensis Cossman, 1892	France	
Siphonaria paucidigitata Cossman, 1907	France	
Siphonaria penginae Dall, 1893		Taxon inquirendum
Siphonaria polygona (Michelotti, 1847)		
Siphonaria revillaria Kiel & Bandel, 2001	Genova, Italy	
Siphonaria spectabilis Deshayes, 1861	France	
Siphonaria tournoueri Vasseur, 1881	Genova, Italy	
Siphonaria vasconiensis Michelin, 1831	Genova, Italy	
Siphonaria vulcanica Harzhauer, Landau & Breitenberger, 2017	Hungary	
Siphonaria wieseri Wade, 1926	Tenneseee, USA	
Siphonaria xinglongtaiensis Youluo, 1979		

Appendix 4.2B

List of references consulted for geographic distribution of *Siphonaria* species: numbering corresponds to that used in Appendix 4.2A to cite references.

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Chapter 5
Synthesis and Conclusion

The central focus of this thesis has been an elucidation of the taxonomy and phylogeny of the genus *Siphonaria* – a group that is globally widespread and plagued by taxonomic confusion. In particular, the thesis focuses on the southern African species and their integration into a global phylogeny. Because the species can be difficult to identify morphologically due to phenotypic plasticity of the shell features, the use of molecular techniques for species identification has been a welcome additional tool for identifying and distinguishing members of the group. After an introductory chapter, the thesis has three substantive sections, the aims and conclusions of which are outlined below, and ends with this synthesis.

Resolution of the status of east-coast southern African species

Southern Africa is home to eleven *Siphonaria* species, of which eight have been surrounded by taxonomic confusion. Specifically, the distinction between *S. concinna* and *S. oculus,* due to identification errors, and the differences among the species in the '*Siphonaria nigerrima* complex' that occur on the east coast of South Africa and southern Mozambique needed to be addressed. Added to that, one species, *S. nigerrima*, has recently been proposed to encompass four previously recognized east-coast species (Teske *et al.* 2007), in contrast to earlier work by Chambers & McQuaid (1994) which had retrieved them as separate species.

In Chapter 2, I was able to clarify the confusion among these species. I established that *S. concinna* and *S. oculus* are indeed separate species on the basis of both genetics and shell morphology. I also resolved that within the *S. nigerrima* complex, *S. tenuicostulata* and *S. anneae* should be resurrected but that the anti-cryptic *S. dayi* remains subsumed under *S. nigerrima*. I also detected the existence of one further unidentified species from a site in southern Mozambique (*Siphonaria* nov. sp. 1) and another possible unidentified species from Mkambati in the Eastern Cape (*Siphonaria* nov. sp. 2). To add to the confusion, the name *S. carbo* is recognized in many data bases as the valid name for members of the *S. nigerrima* complex. From an examination of the type specimen, I was able to conclude that *S. carbo* cannot be considered as an appropriate name for any southern African members of the complex.

I also examined original descriptions and type specimens of the South African species, to establish which names are no longer valid, and tabulate errors that currently exist in websites and databases, including erroneous names attached to genetic sequences in GenBank. I was thus able to provide a list of names that I consider can be validly applied to South African species. I conclude there are 10 valid species in South Africa, and an additional undescribed species in southern Mozambique, bringing the total southern African species up to 11.

Genetic relationships between populations of Siphonaria compressa

In Chapter 3, I address the relationship between two populations of what have thus far been considered a single species: the range-restricted and critically endangered endemic species *S. compressa*. Prior to my thesis there had been no molecular research done on the relationships or specific status of the two isolated *S. compressa* populations. On the basis of my mitochondrial gene analysis, I resolved that the two populations constitute distinct species, which last shared a common ancestor ca. 730 000 years ago. There are no shared haplotypes in the two maternally inherited sequences analysed implying no gene flow between them and no evidence of migration between the two populations. They thus likely have a long history of isolation from each other.

Multivariate analyses of 13 morphometric variables using Canonical Discriminant Analysis revealed clear and substantial differences in shape and size of the shells between the two lagoons and established that the two populations were morphologically significantly different. There were clear differences in shell shape, size and weight between the two populations and they can be separated visually – in particular by a diagnostic difference in shell height/shell length ratio. I recognize that size per se is not a sufficient means of distinguishing species because factors such as temperature and productivity can influence size (as Teske *et al*. 2007 have demonstrated for the tick shell *Nassarius kraussianus*), but the differences in proportions, shapes and colours of these sister species are distinctive and support the genetic evidence that they are sister species. In a future publication I will describe and name this new species but for the purposes of this thesis it is referred to as *Siphonaria* nov. sp. 3.

The fact that the two populations are separate species has important conservation implications. It is, for example, no longer possible to consider using transfers to restore populations should one of them become extinct, which is a distinct possibility given the radical fluctuations in their numbers (Angel *et al*. 2006) due to factors such as flooding or sedimentation. Further exploration of the relationships between these sister taxa and confirmation of the time of their most recent common ancestor could be obtained from next generation sequencing and indeed this has been set in motion. Once these data are analysed a truly complete picture of these endangered endemics will be possible.

Phylogenetic relationships of southern African species in relation to a world phylogeny

No South African species have, to date, been included in any major world phylogeny of the genus *Siphonaria*. The major aim of the fourth chapter of my thesis was to integrate the eleven southern African species I now recognise – and an additional nine species not included in any previously published phylogeny – into one global phylogeny. In doing so, I relied on the most complete phylogeny previously derived for the genus, namely that of Dayrat *et al.* (2014), which concentrated on Indo-West

Pacific species. Many of the groupings previously recorded by Dayrat *et al*. (2014) were retrieved in my phylogeny, most notably the existence of two major clades, A and B. There were, however, specific differences between the two phylogenies in terms of the species that lie successively at the bases of these two clades.

I had hypothesized that the southern African species would be monophyletic, but this proved not to be the case. With one exception, the South African species did all occur in clade A – the exception being *S. serrata*, which unexpectedly fell in clade B. Of the remaining species, all except one sister pair of species did fall within one clade within clade A. Thus, eight of the eleven southern African species are genetically united. It is important to note that this clade is not confined to southern Africa, as *S. asghar*, *S. pectinata* and *S. naufragum* occur elsewhere. The sister pair I allude to comprised *S. compressa* and *Siphonaria* nov. sp. 3 from Knysna, which also fell outside the clade comprising the rest of the South African species, and were more closely allied to an unnamed unit from Oman with similar appearance, and to the Pacific "*normalis*" complex of species and *S. japonica*.

There was a particularly interesting grouping along the Pacific west coast of North and South America, with indications that despite wide geographic separation, the far northern *S. thersites* has close links to the far southern *S. lateralis* and *S. fuegiensis*. *Siphonaria pectinata* and *S. naufragum* – again spatially widely separated as they fall on opposite sides of the Atlantic – form a sister pair, which is in turn the sister clade to the bulk of the South African species. The Australian *S. jeanae*, *S. diemenensis*, *S. funiculata*, *S. tasmanica* and the South American *S. lessonii* are closely related to the South African species. This contrasts with *S. australis* which is clearly different and the sole representative of a clade that is distant from the bulk of the South African species.

Within clade B, there was strong evidence that the central American species *S. gigas*, *S. brannani*, *S. maura*, *S. alternata* and the Indo-Arabian *S. savignyi*, *S. kurracheensis* and *S. belcheri* form a grade at the base of the clade. Thereafter, clade B was separable into two clades, B1 and B2. Two species new to the phylogeny, an unknown species from Japan and the South African *S. serrata*, were retrieved as successively basal to clade B1, the "*atra*" group. Another unknown species that is currently incorrectly identified in Australia as *S. kurracheensis* was recovered as the sister clade to the "*laciniosa*" group and *S. zelandica* (which appears to comprise two geographically separated genetic groups). I suggest *S. javanica*, the type species, falls within this group and is the name that should be applied to unit 25 recognised by Dayrat *et al.* (2014).

Drawing on information provided by my phylogeny and distributional records for the species, I was able to capitalize on the widespread occurrence of the genus to use it as a model to explore ecological correlates such as geographic range relative to mode of larval development, and variations in species diversity relative to latitude.

As I hypothesized, I was able to discern strong latitudinal trends in species richness within the genus, with high diversity present in the lower latitudes, in accord with data for many other marine taxa (Willig & Presley 2018), but with a

marked dip at the equator – which I attributed to the limpet shell form being poorly adapted for the stresses of tropical conditions (Vermeij 2017). I also found that species with direct larval development had significantly smaller latitudinal and geographic ranges per species than those with planktonic development, which is explicable in terms of their reduced dispersal ability (Chambers & McQuaid 1994).

I compared my phylogeny with the subgenera and 'sectia' proposed by Hubendick (1946) and conclude that although there is considerable correspondence between his divisions and the clades I detected, there are also substantial differences. Like Dayrat *et al.* (2014) I showed that Hubendick's two subgenera are not natural monophyletic entities. Two of Hubendick's sectia, *Kerguelenia* (now named *Kerguelenella*) and *Patellopsis* do correspond largely with definable clades. Nevertheless, I advocate retaining the genus *Siphonaria* to incorporate all species currently recognised in the genus, rather than introducing names that will divide the genus. While there may be future merit in erecting names that reflect phylogenetic divisions (particularly between clades A and B), it would be premature to do so until a larger proportion of the species has been examined genetically and placed within the phylogeny.

My addition of extra species to the phylogeny proposed by Dayrat *et al.* (2014) substantially expands the world phylogeny, with the inclusion of the 11 southern African species now recognized, and 9 other species or genetic units from other parts of the world. I was also able to resolve the names of six additional species listed by Dayrat *et al.* (2014) as unnamed genetic units. Despite this, there are clear gaps that still exist in the phylogenetic analysis of the genus. Of the 114 probably valid species or genetic units listed in Appendix 4.2, only 50 were incorporated in my phylogeny. There is also a clear geographic gap in coverage in Brazil, and between central Mozambique and Kenya, where no, or few, specimens have been analysed genetically.

Looking further afield, it would be valuable to explore the members of clade A1 spanning the eastern Pacific coastline of North and South America from *S. thersites* in the far north to the group of currently recognised Southern Ocean species (*S. lateralis*, *S. fuegiensis* and *S. lessonii*) and to resolve their relationships in more detail. A further area requiring more research would be the basal grade in clade B and thus further investigation of the position of *S. gigas*. This would clarify the current confusion among *S. crenata*, *S. belcheri*, *S. savignyi* and *S. kurracheensis* and possibly explain the link between them and the central American species, *S. maura*, *S. alternate* and *S. gigas*. Finally, teasing out the relationships in the "*normalis*", "*laciniosa*" and "*atra*" groups would throw some light on the connection between the South African endemic *S. compressa* clade and the "*normalis*" group to which it is most closely related.

My research is important for a number of reasons. It matters now when the unpredictability of climate change and sudden extreme weather fluctuations generate catastrophic events such as hurricanes and flooding which can cause extinction events. Rising temperatures, too, could have a negative impact, particularly on intertidal life. It specifically contributes to conservation management

in the case of the *S. compressa* complex, and uncovers that there is more undocumented diversity on the southern African east coast than previously suspected. Finally, it adds a substantial component to the global phylogeny of the genus and to the placement of South African species.

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